Binge Alcohol Drinking Exacerbates Ulcerative Colitis Flare

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BINGE ALCOHOL DRINKING EXACERBATES ULCERATIVE COLITIS FLARE

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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PROGRAM IN INTEGRATIVE CELLULAR BIOLOGY

BY
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CHAPTER ONE
INTRODUCTION

Over 1.4 million Americans have been diagnosed with Inflammatory Bowel Disease (IBD), and ulcerative colitis (UC) makes up approximately half of those diagnoses. IBD remains a prominent medical issue not only because of the sheer number of Americans presenting with the disease each year, but also because the exact mechanisms behind disease flare onset remain elusive. Unlike some other chronic diseases, UC cycles between periods of remission and flare. Periods of disease remission can unexpectedly and seemingly without cause turn into periods of active disease – an UC flare, which is characterized by intense abdominal pain, increased weight loss, intestinal inflammation, rectal bleeding, and dehydration. Reappearance of UC symptoms during a flare stem from increases in intestinal inflammatory cytokines of IFN-γ, IL-1, IL-6, and TNF-α, which can lead to a dysbiosis of the colonic microbiome perpetuating the UC flare period. No cure exists for UC. So, patients must obtain symptomatic relief through the use of drug and/or surgical therapies. Management of UC requires patients to avoid stress, certain foods, and alcohol, as all three can potentially induce flare periods of UC.

Interestingly, a widespread recommendation to IBD patients for avoidance of a flare period is ‘Don’t Drink Alcohol’ as some recent work has correlated alcohol consumption with increased GI symptoms in patients with IBD. Alcohol alone not only induces a systemic pro-inflammatory response, but can also be directly harmful to gut barrier integrity. However, how alcohol could result in the exacerbation of UC in murine models of colitis has yet to be
elucidated. Understanding how alcohol consumption exacerbates UC flare will allow for therapeutic intervention to not only prevent UC flare, but also improve the quality of life in patients suffering from UC.

On the other hand, UC remission is associated with periods of asymptomatic disease, which relies on elevated levels of IL-22 to mediate intestinal tissue repair mechanisms. IL-22, a member of the IL-10 family, is a unique cytokine in that it is produced by a number of different immune cells. Its receptor is only found on cells of non-hematopoietic origin, such as epithelial cells. Once bound to its receptor, IL-22 has been shown to signal through several different downstream molecules, however, the signal transducer and activator of transcription factor-3 (STAT3) is the best described. IL-22 has many functions when present in the intestine, which include promoting mucus secretion, enhancing epithelial stem cell proliferation, and increasing anti-microbial peptide secretion from intestine epithelial cells (IECs).

This led us to our central hypothesis that alcohol consumption alters IL-22 release and subsequently impairs defense mechanisms following DSS-induced colitis, thus perpetuating UC flare. To test our central hypothesis, we first generated a murine model of binge alcohol consumption following a DSS-induced colitis flare. To address current gaps in the field of alcohol and colitis we designed three specific aims. **Aim 1** examines whether binge alcohol consumption exacerbates UC flare and characterizes immune cell IL-22 responses to binge alcohol after DSS-induced colitis. **Aim 2** evaluates whether gut barrier breakdown in DSS-colitis is further impaired with alcohol exposure. Finally, **Aim 3** elucidates whether IL-22 normalizes gut barrier integrity after DSS-colitis and binge alcohol exposure.

To examine how alcohol could exacerbate an UC flare, we utilized a 2% dextran sodium sulfate (DSS) solution in the drinking water of mice followed by a 3-day binge alcohol paradigm.
Increased weight loss, colonic shortening, histopathology by hematoxylin and eosin (H&E) staining, and large intestine inflammation by ELISA. Large intestine lamina propria (LP) cells were isolated and analyzed for immune cell IL-22 production. We administered recombinant mouse IL-22 via intraperitoneal (i.p.) injection or the probiotic, *Lactobacillus delbrueckii*, to assess the role of IL-22 and its signaling through STAT3 in mediating protection against perpetuation of UC flare by binge alcohol. Inoculation with *Citrobacter rodentium* following colitis and alcohol was used to elucidate enhanced susceptibility to infection.

Together, these studies give insight into how the intestinal barrier is damaged on a molecular level following alcohol and colitis, and the mechanism by which IL-22 is protective to the intestine following the combined insult. Our findings present a potential new option for UC patient therapy and may also translate to others with conditions that damage the intestinal barrier.
CHAPTER TWO

REVIEW OF RELATED LITERATURE: EFFECTS OF ALCOHOL AND ULCERATIVE COLITIS ON THE INTESTINAL IMMUNE BARRIER AND MICROBIOME

Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) consists of two main chronic disorders: Crohn’s Disease (CD) and Ulcerative Colitis (UC). Incidence and prevalence of both CD and UC have steadily risen in nations such as North America and Europe since they were first described in 1932 and 1859, respectively. However, the adoption of a ‘western’ type lifestyle in Asian countries has led to an even larger global increase in IBD diagnoses\textsuperscript{16}. Evidence supports a combination of genetic susceptibility, environment, intestinal dysbiosis, and/or over-activation of intestinal immune cells being responsible for IBD pathogenicity\textsuperscript{2,5}. At present, no cure exists, which emphasizes the need for further study of IBD and more specifically IBD pathogenicity.

Intestinal Homeostasis

Before discussing the details of how alcohol and colitis affects the intestines, it is important to first understand the structure and functions of the tissues and cells within the intestinal tract. The spatial relationships established between the lumen and barrier of the gut are absolutely essential for the proper function of the gastrointestinal (GI) tract in digestion and nutrient absorption. The GI tract is a continuous tube that begins at the stomach and ends at the anus. The focus of this research is confined UC and, thus, to the large intestine (colon). The colon is composed of four regions proximally to distally: the ascending, transverse, descending and sigmoid colon, respectively, and terminates with the rectum and anus. The large intestines
are held in place to prevent twisting by the mesentery, which also contains the mesenteric lymph nodes (MLNs). The small and large intestines at the histological level contain a barrier of mucous and epithelial cells that block the translocation of lumenal bacteria to extra-intestinal sites. Just below the intestinal epithelia lies a layer of loose connective tissue, the lamina propria (LP), which connects the surface mucosal epithelium to the basement muscularis mucosae. The LP also contains a large number of the intestinal immune cells. The immediate proximity of the intestinal immune cells and the bacteria within the lumen present a major challenge for homeostatic regulation. Thus, the interactions between the immune cells, intestinal barrier, and the lumenal microbiome are of major interest in all areas related to pathology associated with the intestines.

The intestinal physical barrier consists of a layer of mucus and epithelial cells that line the lumen of the intestines, and provide a crucial first line of defense against pathogens. Starting from the lumen, the first component of the physical barrier is a mucus layer. Mucus provides protection from the luminal bacterial content, and also lubricates the intestinal walls for passing bile\textsuperscript{17-20}. Immediately below the mucus inflammatory host immune cells with lumenal bacterial antigens. Disruptions in either the intestinal mucus or epithelial barrier can result in pathogenic bacterial translocation, which can lead to systemic infections, sepsis, and multiple organ failure, underscoring the importance of maintaining barrier integrity\textsuperscript{21-23}. The mucus layer is a key component of the physical barrier of the intestine, and is formed by a glycoprotein, mucin (mainly mucin-2 in rodent intestine), which is secreted by goblet cells found in the intestinal epithelial layer\textsuperscript{24}. Mucin contains a glycosylated peptide backbone, which causes the mucus layer to be incredibly viscous and effective at preventing pathogen penetration\textsuperscript{25}. The mucus layer is
not impenetrable however, and the tight junction complexes between the epithelial cells below the mucosa play a crucial role in providing a second level of protection.

The intestinal wall is lined with a layer of columnar epithelial cells that serve many functions key to maintaining gut homeostasis. First, intestinal epithelial cells (IECs) aid in the absorption and metabolism of nutrition ingested by the host\textsuperscript{26}. IECs contain microvilli on their apical surface to drastically increase the surface area of the intestines, which aid in their absorptive capacity\textsuperscript{27}. IECs also maintain a barrier between the lumen of the intestine and extra-intestinal sites. While many epithelial cells serve these primary roles, there also exist several populations of specialized IECs. Goblet cells are mucus-secreting cells that lay down the mucin layer that lines the intestinal lumen\textsuperscript{24}. Paneth cells are specialized anti-microbial peptide (AMP) secreting cells that aid in regulating the microbiome\textsuperscript{28-30}.

Finally, enteroendocrine cells are a diffuse population of cells that play a major role in sensing the intestine luminal content. These cells have apical physiochemical receptors that signal the release of peptides and hormones from their basolateral surfaces to mediate autocrine and/or paracrine signaling\textsuperscript{31}. All of these specialized epithelial cells must be renewed during the turnover of intestinal epithelial cell generation. Tight junctions are multi-protein complexes consisting of transmembrane, scaffold, and adaptor proteins, and play an indispensable role in the maintenance of barrier function\textsuperscript{32}. The proteins of tight an indispensable role in the maintenance of barrier function\textsuperscript{32}. Tight junctions form a paracellular seal and function as a selectively permeable barrier between adjacent epithelial cells.

Tight junctions consist of several transmembrane proteins including occludin, junctional adhesions, and claudins\textsuperscript{33}. While the function of occludin is currently unknown, it is understood that it does not mediate tight junction formation, but appears to be instrumental in the regulation
of the junctions\textsuperscript{18,34,35}. Claudins are a family of proteins that are both tissue and cell type specific, and are considered to be the main structural components of the tight junctions.

In addition to the physical barrier, the intestine requires an immune barrier, which must distinguish between commensal and pathogenic bacteria so that it does not mount a damaging autoimmune inflammatory response. The immune cells that carry out these tasks comprise parts of both innate and acquired immune functions. They can be found in all areas of the intestines, especially in regions of gut associated lymphoid tissue (GALT). GALT includes the gut epithelium, PPs, MLNs, and LP\textsuperscript{36,37}. Intestinal T cells are found in GALT sites and exist closely with antigen presenting cells (APCs), such as DCs and macrophages, that aid in T cell differentiation and activation. T lymphocytes form a large part of the adaptive immune response in the intestine. Under homeostatic conditions, the balance between inflammatory and immunosuppressive T cells is maintained through cell-to-cell cytokine signaling. Although the intestines contain a large and diverse population of T lymphocytes, the major subsets of resident T cells within the gut include Th1, Th2, Th17, and T-regulatory (Treg) cells\textsuperscript{38}. The default T cell response in the intestines under normal conditions is immunosuppressive. This occurs through the production of TGF-\(\beta\), primarily by APCs, which drives Treg development. In addition to TGF-\(\beta\), IL-4 production drives Th2 cell development and B cell IgA antibody production. IgA also maintains gut homeostasis, in part by regulating the microbiome\textsuperscript{39}.

**Inflammatory Bowel Disease Pathogenicity**

Epidemiological evidence for a genetic contribution in risk of IBD diagnosis has been identified. The largest genetic association study, which employed genome-wide association data for over 75,000 patients and controls, identified 163 susceptibility loci for IBD. Of these 163 loci, 110 conferred risk to both IBD subtypes, whereas 30 loci where unique to CD and 23 loci
were unique to UC. More recently, a trans-ethnic analysis including over 20,000 individuals of European and non-European ancestry identified an additional 38 new IBD loci, highlighting shared genetic risk across populations and increasing the number of known IBD risk loci to 200\textsuperscript{40-42}.

Genetic predisposition, however, cannot be solely responsible for disease etiology, as genetics cannot account for the rapid rise of IBD incidence in certain geographic regions\textsuperscript{43,44}. Several environmental factors, such as smoking, appendicitis, oral contraceptives, diet, breastfeeding, infections/vaccinations, antibiotics, helminths, and childhood hygiene, have been implicated in the increased worldwide incidence of IBD\textsuperscript{45}. These factors pose serious risks, but are limited in their scope as they do not address microbial changes or break in tolerance of intestinal immune cells that could be contributing to IBD diagnoses. Thus, further studies are necessary to better understand the environmental causes of IBD.

A general consensus exists that IBD is associated with compositional and metabolic changes in the intestinal microbiota. Multiple studies have documented differences in the composition of the gut microbiota between patients with IBD and healthy individuals, particularly with respect to microbial diversity and the relative abundance of specific bacterial taxa. Both expansion of potential pathogens and global changes in composition have been described. Species from the phylum, \textit{Firmicutes}, specifically \textit{Faecalibacterium prausnitzii}, are often reduced in the feces of patients with Crohn's disease\textsuperscript{46-55}. Conversely, members of the Proteobacteria phylum, such as \textit{Enterobacteriaceae}\textsuperscript{56,57}, including \textit{Escherichia coli}\textsuperscript{50,58,59}, are commonly increased in patients with IBD relative to healthy individuals. Similarly, a specific association between \textit{Fusobacterium nucleatum}, ulcerative colitis and the development of colorectal cancer has been proposed based on the isolation of a highly invasive strain from
patients with ulcerative colitis. Intestinal inflammation as a consequence of IBD can create an oxidative microenvironment, which can promote the growth of aerotolerant taxa such as Proteobacteria and Actinobacteria. Interestingly and in regards to our laboratory’s current studies, the mouse pathogen, *Citrobacter rodentium*, has been shown to gain a fitness advantage by promoting epithelial aerobic respiration and increasing oxygenation of the mucosal surface. Alterations in intestinal microbial composition have long been associated with chronic inflammation, but it is important to recognize that the relationship between dysbiosis and IBD is probably complex and dynamic, rather than one of simple cause–effect. Dysbiosis can promote the growth of invasive pathogenic bacteria and also can facilitate bacterial translocation through the intestinal mucosal barrier to extra-intestinal sites. These two phenomena contribute to the breakdown of epithelial barrier integrity, which is the prerequisite for the activation of the mucosal immune response.

It has been established that the recognition of commensal-derived antigens by the adaptive immune system or its stimulation by the innate immune system play a key role in the pathogenesis of IBD. Dendritic cells (DC) are at the interface between intestinal epithelial cells and T cells. They present antigens to naive CD4+ helper T cells (Th0) and ensure tolerance to commensal flora by promoting differentiation of regulatory T cells (Treg). In times of infection, activated DCs produce pro-inflammatory cytokines and promote the differentiation of effector T cells, which triggers a local persistent inflammation. The interplay between secreted pro- and anti-inflammatory cytokines determines the balance of different types of T cells in the intestinal mucosa. In IBD, an overactivation of DCs was observed at sites of inflammation, which consequently induces a strong differentiation of effector lymphocytes (CD4+ and CD8+) and of other effector cells such as natural killer (NK) and NK T (NKT) cells while abolishing the
production of regulatory cells. Therefore, in active IBD, the balance of Th and Treg cells is skewed to that of increased Th and decreased Treg\textsuperscript{64}. In the mucosa of patients with UC, there is mostly an infiltration of Th2 lymphocytes\textsuperscript{65}. Moreover, others studies have pointed to Th17 lymphocytes contributing to the shift towards effector T cells over regulator T cells populations in IBD\textsuperscript{66} Th17 lymphocytes (CD4\textsuperscript{+} CD25\textsuperscript{−}) are T cells which produce the proinflammatory cytokine IL17, especially in response to the presence of extracellular bacteria.

**Current Definitions of Alcohol Abuse**

Alcohol (ethanol) abuse represents a major source of health and economic burden in society. Misuse of alcohol can take several different forms, as defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA). Binge drinking, the most common form of alcohol misuse, is defined by any pattern of drinking that raises one’s blood alcohol content to 0.08 g/dL or higher\textsuperscript{67}. This is more generally described as four drinks for women, or five drinks for men in a two-hour period\textsuperscript{67}. Chronic alcohol misuse, or heavy drinking, is defined as binge drinking five or more days per month\textsuperscript{67}. Definitions of alcohol use disorders are fluid as researchers and clinicians re-evaluate what constitutes healthy versus unhealthy drinking behavior.

**Intestinal Pathophysiology with Alcohol Use**

Alcohol consumption alone deleteriously affects the intestinal tract as it breaks down the normal physical and immunological barrier provided by intestinal epithelial cells and gut associated lymphoid tissue (GALT), respectively\textsuperscript{10,22,68,69}. Alcohol induced intestinal erosion impairs intestinal absorption leading to increases in diarrhea and intestinal permeability, allowing for leakage of endotoxins into the circulation\textsuperscript{68,70,71}. Ethanol induced increases of both pro-inflammatory cytokines and chemokines have been found in both the small and large intestinal tract following exposure to alcohol\textsuperscript{72}. The intestinal epithelium and gut associated lymphoid
tissue (GALT) create both a physical and immunological barrier restricting the passage of potential harmful toxins, such as those from intestinal bacteria, from the luminal space to extra-intestinal sites\textsuperscript{73,74}. T and B cells, macrophages, dendritic cells of the Peyer’s patches (PP), mesenteric lymph nodes (MLN), and lamina propria (LP) of the gut associated lymphoid tissue (GALT) make up the intestinal immune barrier. The key components of the physical intestinal barrier include tight junctional complexes, adherens junctions, and desmosomes between intestinal epithelial cells (IECs). In particular, tight junctional complexes of the small and large intestine are made up of the proteins: claudin, occludin, and zonal-occludin. These proteins are imperative to the maintenance of the physical intestinal barrier prohibiting translocation of bacteria out of the lumen while allowing the selective absorption of critical nutrients required by the host. Any perturbation to this tightly regulated intestinal barrier could lead to a so called ‘leaky-gut’, with deleterious effects not only at the level of the gastro-intestinal tract, but also allow bacterial endotoxin to penetrate the mucosa and enter systemic circulation. Alcohol consumption alone is known to disrupt the functional and structural integrity of intestinal epithelial cells contributing to increased gut leakiness by a variety of mechanisms\textsuperscript{75-77}. Firstly, as increases in ROS following ethanol exposure contributed to disruptions in hypothalamic homeostasis, ethanol-induced increases in ROS also led to disruptions in intestinal homeostasis. Researchers have attributed increases in gut leakiness following ethanol treatment to increases in oxidative stress, specifically by nitric oxide (NO). At basal levels, NO is involved in maintaining normal intestinal barrier function\textsuperscript{78}. However, when NO is in excess, as is found after chronic exposure to alcohol, it results in barrier disruption culminating in increased gut leakiness\textsuperscript{79}. Secondly, alcohol use can result in intestinal barrier structure defects via damage to the mucosa observed as loss of epithelium at the apexes of villi, hemorrhagic erosions, and
hemorrhage in the lamina propria\textsuperscript{80}. Thirdly, the human intestines are home to an estimated 100 trillion bacteria\textsuperscript{81}. These microbes are involved in functions including digestion, metabolism, and development of host-immune defense. While the intestinal microbiome of an individual varies depending on a multitude of factors (diet, age, race, geographic location, etc.), the general balance of the major phyla that make up the majority of the intestinal microbiome is fairly similar. Not surprisingly, new technologies such as deep 16S ribosomal sequencing have allowed scientists to gain a much better understanding of the makeup of the intestinal microbiome, and how it varies between a healthy individual and during a diseased state\textsuperscript{81}. Alcohol use has been demonstrated by a number of groups to drastically change the makeup of the intestinal microbiome\textsuperscript{82}. Specifically, data show a general reduction in the ratio of the most prevalent phyla, Firmicutes and Bacteroidetes groups, which generally make up about 85-90\% of the bacteria within the gut\textsuperscript{82-84}. Additionally, a relative increase in the ratio of Gram-negative Proteobacteria occurs following both acute and chronic alcohol consumption, which may lead to increased inflammation in the intestines and other sites following intoxication\textsuperscript{82-84}. It is important to note that not all alcohol consumption is detrimental to microbial populations within the intestine. A study performed by Queipo-Ortuno et al. showed that people that consumed large glass of red wine (272 mL) every day for 20 days had significantly increased \textit{Proteobacteria, Fusobacteria, Firmicutes} and \textit{Bacteroidetes}. Conversely, those who consumed gin (100 mL) per day had a significant decrease in the same phyla\textsuperscript{85}. These data suggest that both the amount and type of alcohol consumed can influence the intestinal microbial communities and this may have a significant impact on the stress and inflammatory response.

This dysbiosis compounded with the destructive nature of alcohol on the integrity of the intestinal epithelium could allow for bacterial translocation out of gut and into the circulation
resulting in systemic inflammation. Finally, alcohol has a stimulatory effect on neuroendocrine hormones, such as corticotropin-releasing hormone (CRH), which can directly lead to increased gut permeability. Increases in CRH within the gut microenvironment can lead to degranulation of mast cells. This degranulation triggers the synthesis and paracrine-like secretion of mediators to gut epithelial cells resulting in epithelial cell F-actin rearrangements increasing gut permeability.

These increases in gut permeability following alcohol consumption allow both bacteria and bacterial products including LPS to translocate outside the lumen and into the circulation. LPS that has breached the gastro-intestinal barrier induces the synthesis and release of IL-1β from mononuclear myeloid cells into the circulation. Furthermore, circulating LPS has been found to increase the pro-inflammatory cytokine, TNF-α, in brain, liver, and serum after one hour. As a compensatory mechanism to increases in pro-inflammatory cytokines, there exists anti-inflammatory cytokines such as IL-10, which is known to down-regulate expression of Th1 pro-inflammatory cytokines. However, when LPS leaks into the circulation as result of increased gut permeability following exposure to alcohol, IL-10 levels are decreased both in brain and intestinal tissues.

Therefore, the addition of alcohol could further perturb the already disrupted intestinal barrier in UC, acting to shift a state of disease remission to that of an active disease UC flare period.

**Ulcerative Colitis and Alcohol Use**

Doctors are recommending limitations on UC patient’s diets, such as refraining from certain foods, and, in the context of our lab’s research interest, refraining from alcohol. Doctor’s
recommendations of avoiding alcohol are based in the idea that alcohol could potentially worsen UC symptoms. However, the scientific evidence in support of this idea is severely lacking.

Regardless of whether or not patient’s heed this advice, they are prescribed medications designed to firstly push their UC into a state of remission, and then secondly to maintain the asymptomatic remission state. Drugs such as 5-ASA, Infliximab, and/or antibiotics or some combination thereof are three of the most commonly prescribed to UC patients. Both 5-ASA and Infliximab are anti-inflammatory through PPARγ activation and anti-TNFα, respectively. Treatment of UC with broad-spectrum antibiotics, such as metronidazole and ciprofloxacin, are used to suppress symptoms of diarrhea and fever caused by intestinal infections.

Of the few studies that have been conducted on IBD and alcohol, three have found a correlation of IBD and alcohol use. The first by Jowett et al. was an observational prospective study in which patients with UC remission were followed for one year to determine the effect of diet on relapse. Patient symptoms were assessed by a validated disease activity index and self-reported dietary habits, such as alcohol use. and the outcome was either clinical relapse or continued remission over the year. They found that patients who drank alcohol during a period of IBD remission experienced increased incidences of relapse into active disease.

The second was done in a cohort of 129 patients with IBD: 52 with Crohn's disease, 38 with ulcerative colitis, and 39 with irritable bowel syndrome (IBS). All participants completed multiple questionnaires. The first was on disease activity (the Crohn's disease activity index or ulcerative colitis clinical activity index, respectively), and a second questionnaire to quantify alcohol consumption by National Institute of Alcohol Abuse and Alcoholism criteria. Lastly, patients were given a questionnaire designed by the researchers to access patients' perception of the effect of alcohol on their GI symptoms and on overall GI symptom severity. This study
found that 75% of patients with active IBD reported worsening of their gastrointestinal symptoms upon drinking alcohol$^69$.

Another large study by Hsu et al. reported that of the 300,000 patients without IBD they retrospectively analyzed over a 10-year period, patients who were at some point hospitalized for alcohol use had a 3.17 fold increase in risk of being diagnosed with IBD compared to the non-alcohol intoxication cohort$^96$. Taken together, these three studies act as strong correlative evidence between alcohol and risk of UC.

Conversely, another group of researchers at the Imperial College of London performed a prospective study on EU citizens, 198 UC and alcohol cases/792 controls and 84 CD and alcohol cases/336 controls were included. Results from this group show no association between risk of IBD and alcohol use$^97$. However, Bergmann et al. only surveyed alcohol consumption at the time of enrollment, and the habits of patients could have changed over the course of the study.

Due to the contradictions between these studies, we sought to establish a mouse model of UC and binge alcohol consumption, thus allowing us to fully elucidate alcohol’s potential role in risk of UC.

**IL-22 Responses in IBD**

During times of recovery into asymptomatic UC remission, colonic IL-22 expression is induced in attempt to maintain intestinal homeostasis$^{98-100}$. IL-22 is a member of the IL-10 cytokine family, and is produced by many immune cells including CD4+ T-cells (specifically Th-17 and Th-22 subsets), γδT-cells, natural killer (NK) cells, innate lymphoid cell class III (ILC-3), and lymphoid tissue inducer (LTi)-like cells$^{101,102}$. The release of IL-22 from T-cell subsets is dependent upon the transcription factor aryl-hydrocarbon receptor (AhR). Recent
studies have also demonstrated that IL-22 secretion from Th-17 cells is dependent upon the presence of IL-23, but not IL-17\textsuperscript{103}.

IL-22 is unique in that its receptor expression is relegated to cells of non-hematopoietic origin, such as IECs\textsuperscript{103}. The IL-22 receptor is a heterodimer composed of an IL-10Rβ and an IL-22R subunit, and is highly expressed within the intestines. Upon binding to its receptor, IL-22 mediates many different effects in the intestines from enhancing mucus secretion in goblet cells\textsuperscript{104}, to promoting anti-microbial protein release from Paneth cells\textsuperscript{105}, to upregulating IEC proliferation\textsuperscript{106}.

Downstream signaling of the IL-22 receptor is mediated mainly through the signal transducer and activator of transcription (STAT)-3 molecule. STAT3 is activated by phosphorylation from Jak1 and Tyk2\textsuperscript{107}. Following phosphorylation, STAT3 forms a homodimer and translocates to the nucleus where it mediates transcription of anti-apoptotic genes such as members of the Bel-2 family\textsuperscript{108}. Understanding, how IL-22 signaling through the phosphorylation of STAT3 will be critical in our elucidation of the mechanism behind which alcohol could potentiate increases in UC symptoms. IL-22 is known to induce intestinal defenses of intestinal epithelial cell (IEC) proliferation and mucus production, increase levels of the anti-inflammatory cytokine IL-10, and induce secretion of antimicrobial peptides (AMPs); all of which could potentially counteract the dysregulation of IEC function, increased inflammation, and dysbiosis present during an UC flare period\textsuperscript{100,109-113}. Therefore, IL-22 is critical for entrance into an UC period of remission, and a recent study found that administration of IL-22 ameliorated UC disease symptoms\textsuperscript{113}. If any IL-22 mediated intestinal repair mechanisms are impaired during an UC remission period, then an UC flare period could persist. We have recently observed that mice exposed to our model of binge alcohol consumption following DSS-
induced colitis are unable to mount proper colonic IL-22 responses. Such alcohol induced decreases in colonic IL-22 levels could impair IL-22 mediated intestinal tissue repair and defense mechanisms required for maintenance of gut barrier integrity, thus allowing for intestinal bacteria and bacterial endotoxins to gain access to extra-intestinal sites resulting in subsequent complications.

**Probiotics in Treatment of Ulcerative Colitis**

This rationale for probiotics as a viable treatment option for UC patients is 2-fold. First, as discussed above, UC pathogenesis is tripartite: genetic predisposition, microbial dysbiosis, and overactive intestinal immune system. Damage to the intestinal barrier during UC can permit pathogenic luminal bacteria to initiate a mucosal inflammatory response that never ceases. Probiotic treatment could bacteriological milieu of the gut to allow for increases in less pathogenic and more anti-inflammatory bacterial species, which could abrogate mucosal inflammation\textsuperscript{114,115}. The second consideration is that UC is a mucosal disease, so a therapy that works at the level of the mucosa should be beneficial.

While antibiotics tend to be most effective in Crohn’s colitis, ileocolitis, and pouchitis, they are less effective in UC\textsuperscript{116-118}. Probiotics by definition are preparations of living microbial cells that, when ingested, are believed to influence the composition of the gut microbiota and consequently benefit the health of the host\textsuperscript{119,120}. Probiotics have been shown to reduce colitis in animal models\textsuperscript{121-123} and to help treat acute, and maintain remission of, UC in humans\textsuperscript{124-130}. One probiotic, *Escherichia coli* is a nonpathogenic strain of *E. coli* that has been shown to be effective for both inducing remission in patients who have UC and maintaining remission for at least 1 year. Researchers have compared *E. coli* Nissle to 5-ASA, which is the standard treatment
for UC, and *E. coli* Nissle was found to be just as effective as 5-ASA for both inducing and maintaining remission over a 1-year period\textsuperscript{125}.

Probiotics alter the function of the mucosal immune system to make it more anti-inflammatory and less pro-inflammatory; specifically, probiotics can stimulate dendritic cells to make them less responsive and reactive to bacteria within the lumen. Additionally, a probiotic preparation consisting of a mixture of *Bifidobacterium breve*, *B. longum*, *B. infantis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, *L. bulgaricus*, and *Streptococcus thermophiles* (VSL#3), which are eight lactic acid bacterial species (VSL#3) has been reported to be effective in maintenance of remission of UC\textsuperscript{128}.

The probiotic, *Lactobacillus delbrueckii*, has recently been shown to activate the transcription factor aryl hydrocarbon receptor (AhR), which can induce expression of IL-22. Takamura et al. found that treatment with *Lactobacillus delbrueckii* in an experimental model of colitis resulted in amelioration of colitis by activating the AhR pathway. *Lactobacillus delbrueckii* induced the mRNA expression of cytochrome P450 family 1A1 (*CYP1A1*), a target gene of the AhR pathway, which was inhibited by the addition of an AhR antagonist\textsuperscript{131,132}. These data make *Lactobacillus delbrueckii* an exciting therapeutic option to determine whether probiotic treatment could potentially reverse exacerbated UC symptoms as a result of alcohol use.
CHAPTER THREE
GENERATING THE MOUSE MODEL OF BINGE ALCOHOL CONSUPTION FOLLOWING DSS-INDUCED COLITIS, WHICH PERPETUATES UC FLARE SYMPTOMS

Abstract

Inflammatory Bowel Disease (IBD) remains a prominent medical concern affecting over one million people in the United States alone. While IBD’s exact cause remains elusive, the two most common forms of the disease are Crohn’s disease (CD) and ulcerative colitis (UC). UC is a cyclical, life-long illness characterized by disease remission and active disease flares causing symptoms of abdominal pain, increased weight loss, intestinal inflammation, rectal bleeding, and dehydration.

In order to avoid worsening of symptoms, UC patients will often heed doctor’s advice to refrain from drinking alcohol. However, there exists a gap in scientific evidence as to whether this phenomenon of alcohol exacerbating UC symptoms truly does occur. The current study sought to establish a mouse model of UC along with a binge alcohol paradigm in order to advance research in this area. To accomplish this, male C57BL/6 were given either 2%, 3%, or 4% dextran sodium sulfate (DSS) in their drinking water for 7 days. Mice in Sham/control group received water. Additionally, one group of 3% DSS treated mice were given a three-day binge of alcohol via gavage at ~3g/kg. Three hours after the last gavage on day 7, mice were humanely euthanized. Body weight was regularly monitored over the course of the 7 days and colon length was measured on day 7 after sacrifice. Although the first trial of the model was promising in that the addition of alcohol to the group of mice given 3% DSS had increased weight loss compared
to 3% DSS vehicle treated mice, the data was unrepeatable. Therefore, we changed out experimental model to give either a 2% or 3% solution of DSS for only five days, instead of seven. Mice in Sham/control group received water. On day five, mice were further subdivided into two groups: mice receiving only one gavage of alcohol (~3g/kg) on day 7 or a three-day binge of alcohol on days 5, 6, and 7. In all cases, three hours after the last gavage on day 7, mice were humanely euthanized. Body weight was regularly monitored over the course of the 7 days and colon length was measured on day 7 after sacrifice. Regardless of whether mice received DSS at a 2% or 3% concentration, when only one gavage of alcohol was given, there were no significant differences in weight loss or colonic shortening. However, the group of mice receiving a 2% DSS concentration in their drinking water for five days in addition to a binge alcohol paradigm of alcohol gavage for 3 days had significantly increased weight loss (**p<0.001) and colon shortening (*p<0.05) compared to 2% DSS vehicle treated mice.

Increased weight loss and colonic shortening are the two critical parameters in the field of UC research to accurately monitor UC disease severity. As statistical significance was reached with the 2% DSS concentration along with a three-day binge alcohol paradigm for both of these parameters, these data suggest that this was the best applicable mouse model to begin our lab’s research of the hypothesis that alcohol – known to be both pro-inflammatory and directly harmful to gut barrier function – could exacerbate a UC flare period. Body weight was regularly monitored over the course of the 7 days. Colon length was measured and large intestines harvested for histopathology, clinical scores, and quantification of IL-18, IL-1β, IL-6, TNFα, and KC proteins levels via ELISA. Consistent with previous reports, mice treated with DSS began losing body weight on day 5 after treatment as calculated by percent weight change from day 0. On day 6, mice receiving a combined insult of DSS and alcohol lost twice as much
weight compared those mice in the DSS Vehicle group, ~10% vs. ~5% respectively. By day 7, the weight loss in mice receiving DSS and alcohol reached 17% to that of their original body weight as compared to only 12% in DSS Vehicle mice. Gross histopathology scores were significantly increased in the DSS Ethanol group compared to DSS Vehicle. This accompanied a significant decrease in colon length in the DSS Vehicle group of mice (p<0.0001) compared to that of Sham Vehicle mice. Interestingly, the addition of alcohol to the DSS treated mice resulted in a more severe decrease in colon length (p<0.05) compared to DSS Vehicle treated mice. Clinical scores of the DSS Ethanol treated mice trended toward an increase compared to the DSS Vehicle mice.

Furthermore, the levels of the pro-inflammatory cytokines, IL-18 and IL-1β, trended to increase in the large intestine of mice receiving DSS and alcohol. IL-18 increased by ~6 fold in the DSS Vehicle group when compared to Sham Vehicle mice (p<0.01). Adding alcohol to the DSS treated mice, further increased levels of IL-18 to ~10 fold compared to the Sham Vehicle group of mice (p<0.001). IL-1β increased ~80 fold (p<0.0001) in mice receiving DSS Vehicle compared to Sham Vehicle mice, but there was no further increase in IL-1β with the addition of alcohol. Both IL-6 and TNFα increased by ~3 fold in DSS Vehicle, but were not further increased with the addition of alcohol. KC also increased ~3 fold in DSS Vehicle treated mice compared to Sham Vehicle, but the addition of alcohol increased KC to that of ~4 fold compared to Shame Vehicle. These data suggest that alcohol perpetuates a DSS-induced colitis flare period resulting in increased weight loss, colonic shortening, histopathology and clinical scores, and inflammation.

**Introduction**

Inflammatory Bowel Disease (IBD) is diagnosed in over 1.4 million Americans each
year. Yet, the exact mechanisms behind disease onset remain elusive\textsuperscript{1,2}. IBD is subdivided into two clinical categories of either Crohn’s disease (CD) or Ulcerative Colitis (UC), characterized by inflammation of the entire gastrointestinal tract in CD or the large intestine only in UC\textsuperscript{1}. UC accounts for over 700,000 IBD diagnoses and is most prevalent in patients under thirty; the age range where an estimated 70\% of binge alcohol drinking episodes occur\textsuperscript{1-3,5,15}. UC is a life-long disease characterized by cycles of asymptomatic remission and active disease flares. UC patients are subject to symptoms of bloody stool and subsequent anemia, bowel incontinence, and weight loss\textsuperscript{2}.

The precise etiology of UC is still unknown, but research has focused on genetic factors leading to over activation of the immune system with a break in tolerance to commensal intestinal bacteria, environmental factors resulting in dysbiosis of the bacterial population residing in the gut, or some combination thereof that could potentially be contributing to disease onset. As there is no cure for UC, patients are forced into maintenance regimens obtaining symptomatic relief through the use of immunosuppressive drugs, antibiotics, or surgical therapies\textsuperscript{2,12,14}. This maintenance of UC requires patients to avoid stress, certain foods, and alcohol, as all three can potentially induce flare periods of UC\textsuperscript{2,133}. Specifically, physicians recommend UC patients maintain a sober lifestyle\textsuperscript{133}, but there exists a gap in knowledge as to how alcohol intoxication affects UC flare periods.

Alcohol consumption is well known to be both pro-inflammatory and directly harmful to gut barrier function as it breaks down the normal physical and immunological barrier provided by intestinal epithelial cells and gut associated lymphoid tissue (GALT), respectively\textsuperscript{10,22,69,134}. Alcohol alone is known to induce intestinal erosion, which can impair intestinal absorption leading to increases in diarrhea and intestinal permeability\textsuperscript{15}, allowing for leakage of bacteria or
bacterial endotoxins into the circulation\textsuperscript{70,71,134}. Despite this, only a handful of studies have explored the impact of alcohol in the setting of UC. Some research has shown that alcohol has a deleterious role in UC by increasing gastrointestinal symptoms\textsuperscript{69}, inducing a flare\textsuperscript{95} and promoting disease onset\textsuperscript{96}, while one study describes that alcohol has no effect in the onset of UC\textsuperscript{97}. The inconsistency of evidence in either support or contradiction of alcohol’s role in exacerbating UC flare and/or onset prompted us firstly to elucidate whether alcohol is a contributing factor in UC flare.

Thus, we developed a mouse model of binge alcohol consumption following an induced colitis flare to test the hypothesis that alcohol exacerbates an UC flare. We found alcohol exacerbates weight loss, clinical scores, colonic shortening and inflammation, suggesting alcohol as an underlying factor in perpetuating symptoms of IBD.

**Materials and Methods.**

**Induction of DSS Colitis.**

Male 8-9 week old (~23-25g body weight) C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). Briefly, mice received either 2\%, 3\%, or 4\% (wt/vol) DSS (40,000 kDa; MP Biomedicals), *ad libitum*, in their drinking water for seven days in Figures 1, 2, 3. Mice in the Sham group received water only acting as a control. The amount of DSS water drank per animal was recorded and no differences in intake between groups were observed. Mice were weighed every day for the determination of percent weight change. This was calculated as: % weight change = (weight at day X - weight at day 0/weight at day 0) X 100. Animals were monitored clinically for rectal bleeding, diarrhea, and general signs of morbidity, including hunched posture and failure to groom. In figures 4 and on, on day 5, DSS was discontinued and replaced with normal drinking water in both the DSS and Sham/control groups.
Binge alcohol paradigm.

On day 5, mice in both the DSS and Sham/control group were further subdivided into two subgroups: mice gavaged with alcohol (~3g/kg) or mice gavaged with water. Mice in the Ethanol groups received alcohol on days 5, 6, and 7 to mimic a binge alcohol abuse pattern.

Figures 1 – 3 and 5 – 6. In Figures 4 and 5, mice were gavaged with either alcohol (~3g/kg) or water on day 7 only.

The experiments described here were carried out in adherence with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and are approved by the Institutional Animal Care and Use Committee, Loyola University Chicago Health Sciences Division, Maywood IL.

Tissue Staining.

For hematoxylin and eosin (H&E) staining, 2cm of colon tissue closest to the rectum was taken from each mouse and saved in 10% formalin. Tissue was fixed with 10% phosphate buffered formalin, paraffin embedded, sectioned at 5 μm, and stained with hematoxylin and eosin by AML laboratories (Saint Augustine, Florida). Images were taken on an Olympus BX43 Microscope using an Olympus DP26 camera.

Histopathology Scoring.

H&E stained sections were analyzed and scored in a blinded manner by Dr. Xianzhong Ding. Dr. Ding is an Associate Professor of Pathology at Loyola University Chicago and member of my Dissertation Committee. Scoring was based on a modified 0-4 point scale examining exudate, epithelial damage, polymorphonuclear leukocyte invasion, and submucosal edema. The values from each of the 4 categories were added to produce a combined histopathology score for each animal.
**Colon Length and Average Clinical Scores.**

Immediately following euthanasia, colons were excised and length measured. Baseline clinical scores were determined using a modified protocol from Siegmund et al.\textsuperscript{135,136}. Briefly, no weight loss was registered as 0, weight loss of 1–5% from baseline was assigned 1 point, 6–10% 2 points, 11–20% 3 points, and more than 20% 4 points. For stool consistency, 0 points were assigned for well-formed pellets, 2 points for pasty and semifomed stools that did not adhere to the anus, and 4 points for liquid stools that did adhere to the anus. For bleeding, 0 was assigned for no blood by using hemoccult (Beckman Coulter), 2 points for positive hemoccult, and 4 points for gross bleeding.

**Enzyme-Linked Immunosorbent Assay.**

Mice in all four groups were sacrificed three hours after the last gavage on day 7, as seen in Figure 8. Large intestines were harvested and homogenized. The homogenates were analyzed for IL-18 (eBioscience), IL-1B (R&D Systems), IL-6 (R&D Systems), TNFα (eBioscience), and KC (BD Biosciences) using their respective ELISAs per the manufacturer’s instructions. The cytokine levels were expressed per milligram of total protein in the homogenates.

**Statistics.**

Comparisons within groups were analyzed using a one-Way ANOVA with a Tukey post-hoc test. Analysis was done using GraphPad Prism software. A confidence level of $p < 0.05$ was considered statistically significant. Significance is represented throughout the manuscript as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. 
Results

Exploration of current UC literature shows researchers using the chemical, dextran sodium sulfate (DSS), in the drinking water of mice to induce and mimic symptoms of UC. UC researches utilize a concentration of DSS ranging anywhere between 1-5%. As can be seen in Figure 1, we explored a range of DSS concentrations, 2, 3, and 4%, for seven days to understand what works best in our hands and the environment of our animal facility.

![Figure 1](image)

**Figure 1. Mouse Model of Colitis Utilizing a Range of DSS from 2-4% Over the Course of Seven Days Followed by a Three-Day Alcohol Binge.**

We found that increasing concentrations of DSS correlate with increases weight loss and colonic shortening, **Figure 2A and 2B, respectively**. The addition of a binge alcohol paradigm to 3% DSS treated mice significantly increased weight loss compared to 3% DSS Vehicle mice (**Figure 2A**), but not colonic shortening, **Figure 2B**.
Figure 2. Increased Weight Loss and Colonic Shortening with Increasing Concentration of DSS and Addition of Ethanol. A. Increased concentrations of DSS and the addition of ethanol increased weight loss. Percent weight change of animals was determined by the following

B
equation: % weight change = (weight at day X-weight at day 0/weight at day 0)*100. Values are mean ± SEM, n=7-9 animals/group. **p<0.01 3% DSS Ethanol compared to 3%DSS Vehicle, ***p<0.0001 DSS Ethanol compared to Sham Vehicle, ****p<0.0001 4% DSS Vehicle compared to Sham Vehicle by ANOVA on day 7. B. Increased concentrations of DSS and the addition of ethanol increased colonic shortening. Values are mean ± SEM, n=5-6 animals/group *p<0.05 2% DSS Vehicle compared to Sham Vehicle, **p<0.01 3% DSS Vehicle compared to Sham Vehicle, ****p<0.0001 3% DSS Ethanol compared to Sham Vehicle, ****p<0.0001 4% DSS Vehicle compared to Sham Vehicle by ANOVA.

From data in Figure 2, we determined a 3% concentration of DSS for seven days was the best working concentration for our lab to move forward. A 3% concentration of DSS is widely used in the field and very well established. Next, we included proper controls in order to determine the optimal binge alcohol paradigm. Disappointingly, as can be seen in Figure 3A, we were unable to repeat the significant increases in weight loss in 3%DSS Ethanol treated mice compared to 3% DSS Vehicle treated mice we saw in Figure 2A. Colonic shortening in Figure 3B followed the same trends as Figure 2B with no significant increases in colonic shortening with the addition of a binge alcohol paradigm.
A.

![Graph showing weight change over time for different treatment groups.]

- **Sham Vehicle**
- **Sham Ethanol**
- **3% DSS Vehicle**
- **3% DSS Ethanol**
- **4% DSS Vehicle**

B.

![Bar graph comparing colon length across different treatment groups.]

- **Sham Vehicle**
- **Sham Ethanol**
- **3% DSS Vehicle**
- **3% DSS Ethanol**
- **4% DSS Vehicle**
Figure 3. No Significant Differences in Weight Loss or Colon Length in DSS Ethanol Treated Mice Compared to Vehicle. A. Increased weight loss with increasing concentrations of DSS, but not with addition of ethanol. Percent weight change of animals was determined by the following equation: % weight change = (weight at day X-weight at day 0/weight at day 0)*100. Values are mean ± SEM, n=6-9 animals/group. ****p<0.0001 3% DSS Vehicle, 3% DSS Ethanol, and 4% DSS Vehicle compared to Sham Vehicle; ****p<0.0001 3% DSS Vehicle, 3% DSS Ethanol, and 4% DSS Vehicle compared to Sham Ethanol by ANOVA on day 7. B. Increased concentrations of DSS, but not the addition of ethanol, increased colonic shortening. Values are mean ± SEM, n=6-9 animals/group. ****p<0.0001 3% DSS Vehicle, 3% DSS Ethanol, and 4% DSS Vehicle compared to Sham Vehicle; ****p<0.0001 3% DSS Vehicle, 3% DSS Ethanol, and 4% DSS Vehicle compared to Sham Ethanol by ANOVA on day 7.

From these data in Figure 3, we re-evaluated our experimental design. A staple in the field of UC research is significant differences in both weight loss and colonic shortening in order to claim a worsened disease state. Furthermore, the idea that someone in an active UC flare (modeled by us keeping DSS in the drinking water for seven days during alcohol binge) would choose to engage in binge alcohol drinking needed to be addressed. Also, the 3% concentration of DSS was potentially causing too much damage to the intestine to pick up the minute differences alcohol could be contributing to the disease state. So, we added a lower concentration of DSS, a 2% solution, in addition to 3%, in addition to stopping DSS on day 5. Additionally, we sought to determine the optimal binge alcohol paradigm. So, for this experiment we simply employed one gavage on day 7, Figure 4.
Figure 4. Mouse Model of Colitis Utilizing Either 2% or 3% DSS for Five Days Followed by a One-Day Alcohol Binge.

With this model of DSS induced colitis and binge alcohol, no significant differences in either weight loss or colonic shortening were observed with just one gavage of alcohol on day seven in either the 2% DSS treated or the 3% DSS treated mice, **Figure 5A and B.**
Figure 5. No Significant Differences in Weight Loss or Colon Length in DSS Ethanol Mice Receiving One Gavage of Ethanol Compared to Vehicle. A. Weight loss of both 2% and 3% DSS treated mice with one gavage of ethanol. Percent weight change of animals was determined by the following equation: % weight change = (weight at day X-weight at day 0/weight at day 0)*100. Values are mean ± SEM, n=6-9 animals/group. B. Colon length of both 2% and 3% DSS treated mice with one gavage of ethanol.

Therefore, we employed a binge alcohol model of 3 gavages on days 5, 6, and 7 with the same two concentrations of DSS, Figure 6.
Figure 6. Mouse Model of Colitis Utilizing Either 2% or 3% DSS for Five Days Followed by a Three-Day Alcohol Binge.

As can be seen in Figure 7A and 7B, mice treated with 2% DSS for five days and three gavages of alcohol experienced significant increases in both weight loss and colon shortening. Mice in both the 3% DSS (Vehicle and EtOH) treatment groups trended towards increased weight loss and colon shortening compared to the 2% DSS treated groups, but as in Figure 3A and B, the addition of alcohol to these 3% DSS treated mice showed no further increase in either parameter.
Figure 7. Significant Differences in Both Weight Loss and Colon Length in 2% DSS Ethanol Treated Mice Compared to Vehicle, Not 3% DSS Ethanol. A. Increased weight loss with 2% DSS and ethanol, but not 3% DSS and ethanol. Percent weight change of animals was determined by the following equation: % weight change = (weight at day X-weight at day 0)/weight at day 0)*100. Values are mean ± SEM, n=6-9 animals/group. ***p<0.001 2% DSS Ethanol compared to 2% DSS Vehicle, ****p<0.0001 2% DSS Ethanol compared to both Sham Vehicle and Sham Ethanol. ****p<0.0001 3% DSS Ethanol compared to both Sham Vehicle and Sham Ethanol. ns = not significant for 3% DSS Ethanol compared to 3% DSS Vehicle by ANOVA on day 7. B. Colonic shortening increased with the addition of ethanol after 2% DSS treatment but not 3%. Values are mean ± SEM, n=6-9 animals/group. *p<0.05 2% DSS Ethanol compared to 2% DSS Vehicle. **p<0.01 2% DSS Ethanol compared to Sham Vehicle. ****p<0.0001 2% DSS Ethanol compared to Sham Vehicle. ****p<0.0001 3% DSS Vehicle and 3% DSS Ethanol compared to Sham Vehicle by ANOVA on day 7.

Thus, our lab determined the best mouse model of DSS-induced colitis and alcohol to use was a 2% concentration of DSS for five days to mimic symptoms of UC in conjunction with a three day binge alcohol paradigm, which showed preliminary evidence that alcohol exacerbates UC.

Weight loss, histopathology score, colonic shortening, and average clinical score are the
most common experimental observations used to assess UC disease severity. Therefore, we first repeated weight loss or gain in our newly adapted model of UC and binge alcohol. Consistent with previous reports, mice treated with DSS began losing body weight on day 5 after treatment as calculated by percent weight change from day 0. On day 6, mice receiving a combined insult of DSS Ethanol lost twice as much weight compared to those mice in the DSS Vehicle group, ~10% vs. ~5% respectively. By day 7, the weight loss in mice receiving DSS Ethanol reached 17% to that of their original body weight as compared to only 12% in DSS Vehicle mice, Figure 8.

Figure 8. Ethanol Increased DSS-induced Weight Loss. Percent weight change of animals was determined by the following equation: % weight change = (weight at day X-weight at day 0/weight at day 0)*100. Values are mean ± SEM, n=8-14 animals/group. ***p<0.001 DSS Ethanol compared to DSS Vehicle, ***p<0.001 DSS Vehicle compared to Sham Vehicle; ****p<0.001 DSS Ethanol compared to Sham Vehicle by ANOVA on day 7.
To address differences in histopathology between the four experimental groups, sections of colon were taken closest to the rectum, stained via H&E, blinded, and scored by a pathologist. Figure 9A shows gross differences in large intestine morphology after DSS Ethanol treatment compared to all other groups. Inflammatory infiltrate, epithelial damage, and crypt damage are severely increased in the DSS Ethanol mice compared to that of the DSS Vehicle. Furthermore, combined histopathology scores in Figure 9B, show significant increases in DSS Ethanol compared to DSS Vehicle.

![Figure 9A](image)

![Figure 9B](image)
Figure 9. Gross Histological Pathologies Increased Following Combined Ethanol and DSS Treatment. A. Representative H&E stained sections of the colon on day 7 (Top row x100, Bottom row x200). B. Combined Histopathology Score following blinded histological scoring as described in detail in Methods section above. Values are mean ± SEM, n=4-5 animals/group. **p<0.01 DSS Ethanol compared to Sham Vehicle and Sham Ethanol, ; *p<0.05 DSS Ethanol compared to DSS Vehicle by ANOVA.

Increased weight loss and histopathology scores accompanied a significant decrease in colon length in our novel model of UC and binge alcohol. As has been observed by many others in the past, DSS Vehicle treated mice experienced a decrease in colon length compared to that of Sham Vehicle mice. Interestingly, the addition of alcohol to the DSS treated mice resulted in a more severe decrease in colon length compared to DSS Vehicle treated mice, Figure 10A. Clinical scores were obtained by combining weight loss, stool consistency, and blood in stool as described in the methods section above. Data in Figure 10B show that the addition of alcohol to DSS-induced colitis trends toward an increase in the average clinical score compared to DSS.
Vehicle, highlighting alcohol’s detrimental effect on UC flares.

A.

B.
Colonic inflammation is a hallmark symptom of UC. To further delineate how alcohol could be exacerbating an UC flare, levels of large intestine pro-inflammatory cytokines were determined. We hypothesized that alcohol would further increase levels of large intestine pro-inflammatory cytokines following the addition of our alcohol binge paradigm. Our results revealed that in mice receiving DSS Ethanol, IL-18 (Figure 11A), IL-1β (Figure 11B), and KC (Figure 11E) trended to increase compared to DSS Vehicle treated mice. However, the cytokines, IL-6 (Figure 11C) and TNFα (Figure 11D) were not found to be increased in the colons of DSS Ethanol treated mice compared to mice receiving DSS Vehicle.
Figure 11. Increased Colonic Inflammation After DSS-Induced Colitis and Ethanol. Colons were harvested, homogenized, and processed on day 7 for the analysis of inflammatory mediators using respective ELISAs. A. IL-18, B. IL-1β, C. IL-6, TNFα, E. KC by ELISA. Values are mean ± SEM 3-6 animals per group *p<0.05; **p<0.01; ***p<0.001 all groups compared to Sham Vehicle by ANOVA.

Although increases in inflammation following DSS Ethanol did not reach statistical significance, total large intestine homogenates were used for all ELISAs, and we anticipate specifically isolating inflamed areas in contrast to diluting inflamed areas with non-inflamed areas (as commonly occurs in the intestines of UC patients) will yield statistical significance. However, future work will focus on this and the specific cell types in the intestine responsible for the increases in inflammation that we do see in the total homogenates.
Summary

In the current study, we were able to show that neither seven days of 3% DSS in the drinking water of mice plus a three day binge model of alcohol nor a 2% DSS solution with one alcohol gavage on day 7 showed significant differences in the UC symptoms of weight loss and colonic shortening. While it is plausible that alcohol could have been mediating other increases in UC symptoms at the 3% DSS concentration or 2% DSS with one gavage of alcohol, we needed both weight loss and colonic shortening to be statistically significant between ethanol and vehicle treated mice to claim alcohol induces increases in symptoms of UC.

However, we were able to show that a 2% concentration of DSS given ad libitum for five days in the drinking water of mice was sufficient to induce symptoms of UC. Moreover, a binge alcohol paradigm of three gavages on days 5, 6, and 7 exacerbated UC symptoms of weight loss and colon length shortening: 2 of the most common parameters measured in the field of UC to assess disease severity. Evidence from these experiments gave rise to our current and, to the best of our knowledge, novel murine model of UC and alcohol, which has allowed a better understanding of how drinking alcohol could affect UC patients. Understanding potential environmental factors that could contribute to disease flares, either as a trigger or an exacerbation of symptoms, is critical to improving the quality of life of UC patients stuck in the maintenance of their disease hoping to avoid a flare or a worsening of symptoms during a flare period.

We were able to show that mice undergoing a binge alcohol paradigm following DSS-induced colitis had exacerbated symptoms of UC as shown by increases in weight loss, colon shortening, histopathology and clinical scores, and inflammation, all of which are standard assessments of UC severity in mouse models.
CHAPTER FOUR
DECREASED LEVELS OF LARGE INTESTINE INTERLEUKIN-22 PLAY A ROLE IN ALCOHOL INDUCED EXACERBATION OF UC FLARE

Abstract

Ulcerative colitis, one of the two most common forms of IBD, is a disease characterized by cycles of active disease flare and inactive disease remission. During UC remission, IL-22 expression is upregulated, acting as a hallmark of entrance into a UC remission period as it stimulates proliferation, mucous protection, and AMP secretion within the intestine. Recently, we found that in our mouse model of binge alcohol consumption after DSS-induced colitis, alcohol increases severity of UC flare symptoms. In this study, we assessed whether alcohol influenced IL-22 expression and thereby perpetuates UC flare. To accomplish this, male C57BL/6 were divided into two groups: DSS and Sham. In DSS group, mice received 2% DSS ad libitum in their drinking water for 5 days to induce UC. Mice in Sham/control group received water. On day 5, DSS was removed from the drinking water to mimic entrance into remission. Additionally on day 5, DSS and Sham/control group mice were further subdivided into two subgroups: mice gavaged with alcohol (~3g/kg) or mice gavaged with water days 5, 6, and 7. Three hours after the last gavage on day 7, mice were humanely euthanized. Large intestines were harvested and processed for quantification of the cytokines IL-22 and IL-17. Furthermore, large intestine lamina propria immune cells were isolated for assessment of total IL-22+ cells and IL-22+ T cells, innate lymphoid cells type 3, and neutrophils by FACS analysis. Protein levels of large intestine IL-22 were significantly decreased ~6.9 fold (p<0.05) in DSS Ethanol
compared to DSS Vehicle. In contrast, levels of the pro-inflammatory cytokine, IL-17, remained elevated in DSS Ethanol treated mice compared to DSS Vehicle. The percentage of total IL-22+ lamina propria cells in the large intestine was significantly increased in DSS Vehicle treated mice compared to DSS Ethanol. No differences in IL-22+ CD3+CD4+ T cells, NKp46+ innate lymphoid cells type 3, or GR1+ neutrophils were observed between the DSS Vehicle and DSS Ethanol group. Examination of IL-22+γδ T cells, however, revealed that DSS Vehicle treated mice had a significantly increased percentage of IL-22+γδ T cells, while IL-22+γδ T cells from DSS Ethanol treated mice were unable to mount this IL-22 response. Failure to mount the IL-22 mediated repair response needed for entrance into remission in alcohol treated mice could be a potential explanation for the exacerbated UC flare period.

**Introduction**

Inflammatory bowel disease (IBD) is an idiopathic gastrointestinal disease of chronic inflammation that takes over the intestinal mucosa, and thereby destructs both the structure and function of the gastrointestinal tract. IBD encompasses a multitude of GI inflammatory conditions, but the two most prevalent are Crohn’s Disease (CD) and Ulcerative Colitis (UC). CD affects the entirety of the GI tract, while UC is restricted to the colon with inflammation emanating from the rectum and advancing to the rest of the colon. During UC microbial dysbiosis can drive pathogenicity, but there also exists an improper mucosal immune response to the altered intestinal microbiota. This dysbiosis has profound effects on the immune system and intestinal health. The altered microbial composition changes the balance between T regulatory cells (Treg) and T helper cells (Th) cells in the lamina propria conditioning an inflammatory state. This inflammatory state is termed active UC flare. UC cycles between active flare periods and periods of asymptomatic disease remission. A recent series of clinical studies have
shown that complete regeneration of the intestinal mucosa, called “mucosal healing”, predicts long-term remission and low risk of surgical treatment in IBD patients.\textsuperscript{139}

Interleukin (IL)-22 is a key cytokine that links intestinal immune activation to epithelial repair and barrier protection following damage.\textsuperscript{103,140} IL-22 is expressed by numerous immune cells, including T helper cells, γδ T cells, type 3 innate lymphoid cells (ILC3), natural killer (NK) cells, and neutrophils. Intestinal epithelial cells express the IL-22R complex, and binding of IL-22 results in the induction of mucins, antimicrobial peptides (AMPs), and anti-apoptotic pathways that collectively aid in limiting bacterial encroachment while promoting epithelial proliferation, wound healing, and repair.\textsuperscript{141} Mice that lack the ability to produce IL-22 following administration of dextran sodium sulfate (DSS) or \textit{Citrobacter rodentium} are grossly unable to repair barrier damage or control pathogenic bacterial expansion.\textsuperscript{100,112,142} These data suggest that IL-22 plays a major function in mucosal barrier defense and repair mechanisms, which could be responsible for entrance into UC remission.

In our model of DSS-induced colitis and binge alcohol, the UC flare-inducing agent, DSS, is removed three days before sacrifice to model entrance into UC remission. Sugimoto, Zenewicz, Monteleone, and others UC researchers have provided evidence that IL-22 protects and inhibits intestinal inflammation in the context of colitis.\textsuperscript{112,141,143} Our results show that DSS Vehicle treated mice can mount the proper IL-22 response within the large intestine to begin repair mechanisms required for UC remission. However, the IL-22 response is disrupted in DSS Ethanol treated mice. Unexpectedly, we found that the potential source of increased IL-22 in the large intestine of DSS Vehicle treated mice is lamina propria γδ T cells, while γδ T cells from mice in the DSS Ethanol group were unable to mount the proper IL-22 response.
Materials and Methods

Induction of DSS Colitis and Binge Alcohol.

Male 8-9 week old (~23-25g body weight) C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). Briefly, mice were randomly separated into four experimental groups: Sham Vehicle, Sham Ethanol, DSS Vehicle, and DSS Ethanol. DSS treated mice received 2% (wt/vol) DSS (40,000 kDa; MP Biomedicals), *ad libitum*, in their drinking water for five days. Mice in the Sham group received water only for 5 days acting as a control. On day 5, DSS was discontinued and replaced with normal drinking water in both the DSS and Sham/control groups. On day 5, mice in both the DSS and Sham/control group were further subdivided into two subgroups: mice gavaged with alcohol (~3g/kg) or mice gavaged with water on days 5, 6, and 7 to mimic a binge alcohol abuse pattern. Mice were weighed every day to determine weight change calculated as: % weight change = (weight at day X - weight at day 0/weight at day 0) X 100.

Enzyme-Linked Immunosorbent Assay.

Mice in all four groups were sacrificed three hours after the last gavage on day 7. Large intestines were harvested and homogenized. The homogenates were analyzed for IL-22 (eBioscience) and IL-17 (R&D Systems). The cytokine levels were expressed per milligram of total protein in the homogenates.

Lamina Propria Cell Isolation from Colons.

Colons were collected aseptically and placed into a collagenase D bath for 15 minutes at 37°C, as described previously. Collagenase D (0.5 mg/mL) was prepared in Hank’s Buffered Saline Solution (HBSS, Fisher Scientific) containing Ca2+ and Mg2+ and supplemented with 10 mM HEPES, 50 μg/mL gentamicin, 100 U/mL penicillin, and 5% fetal
bovine serum. After collagenase D treatment, the separated cell suspension was filtered through a 70-μm nylon filter, and washed and resuspended at a concentration of $5 \times 10^6$ cells/mL in complete media (RPMI 1640 supplemented with 2 mM L-glutamine, 10 mM HEPES, 50 μg/mL gentamicin, 100 U/mL penicillin, and 10% fetal bovine serum).

**Fluorescence Activated Cell-Sorting (FACS).**

For the measurement of large intestine T cell, innate lymphoid cell 3, and neutrophil cell populations mixed cells were re-suspended in fluorescence-activated cell sorting (FACS) buffer (phosphate buffered saline with 5% fetal bovine serum) at a concentration of $1 \times 10^6$ cells/mL. Cell suspensions were blocked with purified antimouse CD16/32 for 20 minutes at 4°C and stained with live/dead PacOrange dye to separate separate live cell populations. Isolated cells were further stained with PerCP – Cy5.5 conjugated antimouse CD3, APC eFluor 780 - conjugated antimouse CD4, APC-conjugated antimouse NKp46, PE Cy7 conjugated – antimouse GR1, FITC – conjugated antimouse γδ TCR and PE-conjugated antimouse IL-22 for 30 minutes in the dark at 4°C. The cells were washed twice and resuspended in 0.5-mL FACS buffer. All samples were analyzed at the Loyola University Health Sciences Division FACS Core Facility using a 7-color flow cytometer (BD FACSCanto) and FlowJo Software (Treestar).

**Statistics.**

Comparisons within groups were analyzed using a two-Way ANOVA with a Tukey post-hoc test. Analysis was done using GraphPad Prism software. A confidence level of $p < 0.05$ was considered statistically significant. Significance is represented throughout the manuscript as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. 
Results

As seen in Figure 12, DSS Vehicle treated mice had elevated levels of IL-22 in large intestine homogenates, an indicator of entrance into UC remission. By contrast, mice in the DSS Ethanol group show decreased levels of IL-22 compared to DSS Vehicle highlighting the continuance of UC flare and inability to enter into UC remission.

![Graph showing IL-22 levels in different groups](image)

**Figure 12. Elevated Levels of IL-22 in DSS Vehicle but Not DSS Ethanol Treated Mice.** Values are mean ± SEM 6-8 animals per group. *p<0.05 DSS Ethanol compared to DSS Vehicle by ANOVA; **p<0.01 DSS Vehicle compared to Sham Vehicle by ANOVA

Large intestinal levels of the pro-inflammatory cytokine, IL-17, however, remain elevated in both the Vehicle and Ethanol treated mice following DSS-induced colitis, Figure 13.
Multiple immune cells within the large intestine lamina propria (LP) are capable of producing IL-22, such as T cells, innate lymphoid cells, and neutrophils. We next sought to determine what specific cells were responsible for the elevated levels of IL-22 we observed in DSS Vehicle mice, and, furthermore, what cell population or populations were impaired in their ability to produce IL-22 following treatment with both DSS and ethanol.

To accomplish this, large intestine lamina propria cells were isolated and FACS sorted. **Figure 14** demonstrates that the percentage of IL-22+ cells in the large intestine lamina propria in DSS Ethanol treated mice is significantly reduced compared to Sham Vehicle. There is a
downward trend of decreased IL-22+ cells in DSS Ethanol compared to DSS Vehicle, but it did not reach significance.

Figure 14. Binge Alcohol Consumption Following DSS-Induced Colitis Decreases the Percentage of IL-22+ cells in Large Intestine Lamina Propria. Values are mean ± SEM 4-6 animals per group. *p<0.05 DSS Ethanol compared to Sham Vehicle.

When large intestine LP lymphoid cells were sorted into CD3+CD4+ T cells, we did not find any difference in their percentage in DSS Ethanol group compared to other DSS Vehicle or sham/control mice. However, the percentage of CD3+CD4+ T cells that were also IL-22+ was significantly decreased in both DSS treated groups, but not significantly different between Vehicle and Ethanol, Figure 15.
Next, we examined a population of type 3 innate lymphoid cells (ILC3s) in their ability to express IL-22 as ILC3s have been implicated in the pathogenesis of ulcerative colitis. We chose CD3-CD4- live lymphocytes, and then utilized the cell surface marker NKp46 to differentiate ILC3s, which are NKp46+. Similar to what other researchers have observed there was a decrease in the percentage of IL-22+ ILC3s following DSS-induced colitis. However, Figure 16 demonstrates that in our model of binge alcohol consumption after DSS-induced colitis there was no difference in large intestine ILC3s ability to produce IL-22 after receiving alcohol compared to vehicle treated mice.
Another cell type known not only to be involved in the pathogenesis of UC, but also involved in IL-22 production is neutrophils. Therefore, we stained our isolated large intestine LP cells with a common neutrophil marker GR1 and examined the percentage of neutrophils also expressing IL-22. We found a significant increase in the percentage of neutrophils infiltrating the large intestine LP following DSS-induced colitis, but there was no difference with the addition of alcohol. Furthermore, the percentage of IL-22+ neutrophils was significantly decreased in the large intestine LP following DSS-induced colitis compared to shams but this
was also not found to be significantly different between the DSS Vehicle and DSS Ethanol treated mice, Figure 17.

Figure 17. No Difference in Percentage of GR1+IL-22+ Neutrophils After Alcohol Consumption in DSS-Induced Colitis. %GR1+. Values are mean ± SEM 6-12 animals per group. ***p<0.001 DSS Vehicle vs. Sham Vehicle and Sham Ethanol; ***p<0.001 DSS Ethanol vs. Sham Vehicle and Sham Ethanol by ANOVA. %GR1+IL-22+.Values are mean ± SEM 6-12 animals per group. **p<0.01 DSS Vehicle and DSS Ethanol vs. Sham Ethanol by ANOVA.

Finally, we assessed γδ T cells, a population of T cells with a distinct T cell receptor (TCR) and known to produce IL-22. Figure 18 shows that the percentage of γδ T cells in the large intestine lamina propria increase in both DSS Vehicle and DSS Ethanol treated mice, but
we did not observe any further increase with the addition of alcohol. Interestingly, the percentage of IL-22+ γδ T cells was significantly increased in DSS Vehicle treated mice compared to both Sham Vehicle and Sham Ethanol, which mimics our results of IL-22 levels in large intestine homogenates in Figure 12. Furthermore, this increase in IL-22+ γδ T cells was decreased in the DSS Ethanol group compared to DSS Vehicle.

Figure 18. The Percentage of IL-22+ γδ T Cells Significantly Increased After DSS-Induced Colitis, but Following Binge Alcohol this Increase Was Impaired. %γδ T cells. Values are mean ± SEM 6-12 animals per group . **p<0.01 DSS Vehicle vs. Sham Vehicle and Sham Ethanol; ***p<0.001 DSS Ethanol vs. Sham Vehicle and Sham Ethanol by ANOVA. % IL-22+ γδ T cells. Values are mean ± SEM 6-12 animals per group . *p<0.05 DSS Vehicle vs. Sham Vehicle and Sham Ethanol by ANOVA.
These data give evidence to the fact that the alcohol induced reduction in large intestine IL-22 following DSS-induced colitis could be attributed to an inability of γδ T cells to mount a proper IL-22 response when alcohol is present, thus exacerbating an UC flare period.

Summary

The goal of this study was to understand how binge alcohol consumption could be perpetuating an UC flare. The cytokine, IL-22, has been found to be a hallmark of entrance into UC remission. We found that the DSS Vehicle treated mice, which were allowed to recover for 3 days, as DSS was removed from their drinking water Day 5, had increased protein levels of IL-22 in large intestine homogenates. After profiling large intestine lamina propria immune cells known to produce IL-22, we found that DSS Vehicle treated mice had a significantly increased percentage of IL-22+ γδ T cells. However, this IL-22 response both at the level of large intestine homogenates and γδ T cells was completely ablated in DSS Ethanol treated mice.

Alcohol’s role in diminishing the IL-22 response needed for entrance into UC remission could potentially explain the exacerbated UC symptoms we have previously observed. Therefore, increasing levels of large intestine IL-22 in the context of binge alcohol consumption and UC could act a potential therapeutic target to improve lives of UC patients.
CHAPTER FIVE

INTERLEUKIN IL-22 ATTENUATES ALCOHOL INDUCED INCREASES IN INTESTINAL DAMAGE FOLLOWING DSS-COLITIS

Abstract

Ulcerative Colitis (UC) results in chronic inflammation and ulcers in the innermost lining of the large intestine and rectum. The disease process is characterized by cyclical periods of active illness and remission, but the exact etiology of UC flare has yet to be elucidated. However, UC remission periods rely on elevated levels of IL-22 to mediate intestinal tissue repair mechanisms. Alcohol alone induces a systemic pro-inflammatory response with destructive effects on the integrity of the intestinal barrier. Our lab recently observed that alcohol further increases symptoms of DSS-induced colitis such as weight loss, colon shortening, and intestinal inflammation. Furthermore, levels of IL-22 were decreased in both large intestine homogenates and isolated lamina propria cells following DSS-induced colitis and alcohol. Therefore, we used two approaches in attempt to restore or induce large intestine IL-22 with the hypothesis that by re-establishing IL-22 we could alleviate the exacerbated symptoms of UC we observe following DSS-induced colitis and alcohol.

First, we administered recombinant IL-22 resulting in four experimental groups: DSS Vehicle, DSS Ethanol, DSS Vehicle + rIL-22, and DSS Ethanol + rIL-22. Mice received DSS for 5 days to induce UC. On day 5, mice were divided into two groups: mice gavaged with alcohol or mice gavaged with water on days 5, 6, and 7. On the evening of day 5, mice were further subdivided into mice receiving rIL-22 at 1mg/kg or saline via i.p. injection. Large intestine
lumenal content was collected and DNA isolated for qRT-PCR analysis of *Enterobacteriaceae*, *Lactobacillus*, and Total Bacteria. IL-22 administration substantially restored weight loss of DSS Ethanol treated mice back to that of DSS Vehicle (~12.5% back to ~6% on day 7). Increased colonic shortening (**p<0.001 DSS Ethanol + rIL-22 vs DSS Ethanol) and increased *Enterobacteriaceae* were also attenuated following binge alcohol and colitis with IL-22 treatment. Knockout of signal transducer and activator of transcription factor-3 (STAT3) in intestine epithelial cells resulted in loss of IL-22 protection, demonstrating STAT3 is required to remediate the exacerbated UC symptoms we observe following binge alcohol.

*Lactobacillus delbrueckii* is a probiotic known to play a role in the activation of IL-22 through the AhR pathway. Therefore, mice were divided into four experimental groups: DSS Vehicle, DSS Ethanol, DSS Vehicle + *Lacto*, DSS Ethanol + *Lacto*. Mice received DSS for 5 days to induce UC. On day 5, mice were divided into two groups: mice gavaged with alcohol or mice gavaged with water on days 5, 6, and 7. The mice were sacrificed 3 hours after the last gavage. Large intestine lumenal content was collected and DNA isolated for qRT-PCR analysis of Gammaproteobacteria, *Enterobacteriaceae*, *Lactobacillus*, and Total Bacteria. Colons were harvested for ELISAs on IL-17 and IL-22 and Western Blots for STAT3 and p-STAT3. Treatment with *Lacto* attenuated both weight loss and colon length in DSS alcohol mice back to levels of DSS alone (p<0.01 and p<0.001, respectively). Additionally, *Lacto* treatment mitigated the large increases in *Enterobacteriaceae* copy number seen in DSS alcohol treated mice. *Lacto* treated mice trended towards a decrease of IL-17 and an increase in IL-22 in DSS alcohol mice. Levels of p-STAT3 were decreased in DSS alcohol treated mice compared to DSS vehicle, but administration of *Lacto* in DSS alcohol mice increased levels of p-STAT3 back to that of DSS vehicle group. Furthermore, treatment with *Lacto* supernatant alone is not sufficient to mitigate
the exacerbation of US following alcohol. Instead, *Lacto* bacteria must be administered in order to see the reversal of increased UC symptoms after binge alcohol. Our findings suggest that *Lactobacillus delbrueckii* contributes to repair mechanisms by increasing levels of IL-22, resulting in phosphorylation of STAT3, thus attenuating the alcohol induced increases in intestinal damage after colitis.

**Introduction**

Inflammatory Bowel Disease (IBD) refers mainly to Crohn’s Disease and Ulcerative Colitis. The etiology of both autoimmune diseases remains unknown. However, evidence supports a combination of environment, genetic susceptibility, intestinal dysbiosis, and/or over-activation of intestinal immune cells being responsible for IBD pathogenicity\textsuperscript{3,148-150}. At present, no cure exists, which emphasizes the need for further study of IBD and more specifically IBD pathogenicity.

UC is a chronic cyclical disease with periods of active disease flares and inactive disease remission. Treatment of UC focuses on keeping patients in the remission state via combination therapies involving anti-inflammatory drugs like Mesalazine (5-ASA), corticosteroids, or various other biologics\textsuperscript{5,14}.

Increased intestinal levels of IL-22 are linked to UC remission. IL-22 protects the intestinal barrier by promoting mucin production, epithelial cell proliferation, and anti-microbial peptide secretion (e.g. Reg3β/Reg3γ)\textsuperscript{151}. Downstream signaling is limited to epithelial cells as the IL-22 receptor, IL-22R1, is only expressed on cells of non-hematopoietic origin\textsuperscript{152}. The primary producers of IL-22 in the intestine are type-3 innate lymphoid cells (ILC-3), and T-helper(Th)-17 and Th-22 cells that reside in gut-associated lymphoid tissue (GALT)\textsuperscript{102}. One way IL-22 mediates its protective effects is through the Janus kinase (Jak)/STAT pathways\textsuperscript{102}. The
activation of STAT3, via phosphorylation, has been shown to be sufficient for IL-22 mediated protection in a variety of systems, including alcoholic liver disease, hepatitis, and graft-versus-host disease.

As the previously mentioned treatments are associated with numerous side effects and discomfort and have yet to be studied in the context of binge alcohol, the goal of this study was to first examine whether administration of exogenous IL-22 could alleviate the alcohol induced exacerbation of UC symptoms we have previously observed. Additionally, the probiotic, *Lactobacillus delbrueckii*, has recently been shown to activate the transcription factor aryl hydrocarbon receptor (AhR), which can induce expression of IL-22. Thus, the second goal of this study was to examine whether therapeutic intervention with the probiotic, *Lactobacillus delbrueckii*, modulated alcohol-induced exacerbation of UC flare.

We further assessed whether administration of IL-22 required STAT3 signaling and whether treatment with *Lactobacillus delbrueckii* activated the IL-22/STAT3 signaling pathway to produce protective effects following alcohol consumption and DSS colitis. We hypothesized that rIL-22 treatment and/or upregulation of IL-22 by *Lactobacillus delbrueckii* would protect DSS Ethanol treated mice against the increased symptoms associated with UC flare via STAT3.

**Materials and Methods**

**Induction of DSS Colitis and Binge Alcohol.**

Male 8-9 week old (~23-25g body weight) C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). Intestine epithelial cell specific VillinCre STAT3$^{\text{flox/flox}}$ knockout (henceforth referred to as “STAT3$^{-/-}$”) mice were a generous gift from Dr. Bin Gao at the National Institute of Alcohol Abuse and Alcoholism (NIAAA), and were re-derived at Jackson Laboratories (Bar Harbor, ME). All groups received 2% (wt/vol) DSS (40,000 kDa; MP
Biomedicals), *ad libitum*, in their drinking water for five days. Mice in the Sham group received water only for 5 days acting as a control. On day 5, DSS was discontinued and replaced with normal drinking water in all groups. On day 5, mice in both the DSS and Sham/control group were further subdivided into two subgroups: mice gavaged with alcohol (~3g/kg) or mice gavaged with water on days 5, 6, and 7 to mimic a binge alcohol abuse pattern. The amount of DSS water consumed per animal was recorded and no differences in intake between groups were observed. Mice were weighed every day for the determination of percent weight change. This was calculated as: % weight change = (weight at day X - weight at day 0/weight at day 0) X 100.

Animals were monitored clinically for rectal bleeding, diarrhea, and general signs of morbidity, including hunched posture and failure to groom.

**Recombinant IL-22 (rIL-22) Treatment.**

Mice were subjected to the DSS-induced colitis and binge alcohol paradigm as described above. However, on day 5 mice were further divided into two subgroups: mice receiving rIL-22 at 1mg/kg (GenScript, Piscataway Township, NJ) via intraperitoneal injection or mice receiving PBS on days 5 and 6. This resulted in four experimental groups: DSS Vehicle, DSS Ethanol, DSS Vehicle + rIL-22, and DSS Ethanol + rIL-22, Figure 19.

![Figure 19. Model of Treatment with rIL-22 in the Context of DSS-Induced Colitis and Binge Alcohol.](image-url)
**Lactobacillus delbrueckii (Lacto) Treatment.**

Mice were subjected to the DSS-induced colitis and binge alcohol paradigm as described above. However, on day four mice were further divided into two subgroups: mice gavaged with 1 x 10^11 CFUs of *Lacto* suspended in 300 mL of PBS or 300 mL of PBS alone on days 4, 5, and 6. This resulted in four experimental groups: DSS Vehicle, DSS Ethanol, DSS Vehicle + *Lacto*, and DSS Ethanol + *Lacto*.

**Figure 20. Model of Treatment with Lactobacillus delbrueckii in the Context of DSS-Induced Colitis and Binge Alcohol.**

**Colon Length.**

Immediately following euthanasia, colons were excised and length measured.

**Lamina Propria Cell Isolation from Colons.**

Colons were collected aseptically and placed into a collagenase D bath for 15 minutes at 37°C, as described previously^144^–^147^. Collagenase D (0.5 mg/mL) was prepared in Hank’s Buffered Saline Solution (HBSS, Fisher Scientific) containing Ca2+ and Mg2+ and supplemented with 10 mM HEPES, 50 μg/mL gentamicin, 100 U/mL penicillin, and 5% fetal bovine serum. After collagenase D treatment, the separated cell suspension was filtered through a 70-μm nylon filter, and washed and resuspended at a concentration of 5 x 10^6 cells/mL in complete media (RPMI 1640 supplemented with 2 mM L-glutamine, 10 mM HEPES, 50 μg/mL gentamicin, 100 U/mL penicillin, and 10% fetal bovine serum).
**Fluorescence Activated Cell-Sorting (FACS).**

For the measurement of large intestine T cell, innate lymphoid cell 3, and neutrophil cell populations mixed cells were re-suspended in fluorescence-activated cell sorting (FACS) buffer (phosphate buffered saline with 5% fetal bovine serum) at a concentration of $1 \times 10^6$ cells/mL. Cell suspensions were blocked with purified antimouse CD16/32 for 20 minutes at 4°C and stained with live/dead PacOrange dye to separate separate live cell populations. Isolated cells were further stained with PerCP – Cy5.5 conjugated antimouse CD3, APC eFluor 780 - conjugated antimouse CD4, APC-conjugated antimouse NKp46, PE Cy7 conjugated – antimouse GR1, FITC – conjugated antimouse $\gamma\delta$ TCR and PE-conjugated antimouse IL-22 for 30 minutes in the dark at 4°C. The cells were washed twice and resuspended in 0.5-mL FACS buffer. All samples were analyzed at the Loyola University Health Sciences Division FACS Core Facility using a 7-color flow cytometer (BD FACSCanto) and FlowJo Software (Treestar).

**Quantitative Analyses of Fecal Microbiome.**

Real-time PCR was used to quantify bacterial ribosomal small subunit (SSU) 16S rRNA gene abundance, as described previously\textsuperscript{[157].} Primers targeting SSU rRNA genes of microorganisms at the domain level (Bacteria), phylum level (Gammaproteobacteria), and at the family level (Enterobacteriaceae and Lactobacillus) were used. Primers included F: (ACTCCTACGGAGGCAGCAGT) and R: (ATTACCGCGGCTGCTGGC) for domain-level analyses, F: (TCGTCAGCTCGTGTYGTGA) and R: (CGTAAGGGCCATGATG) for Gammaproteobacteria, and F: (GTGCCAGCMGCCGCGGTAA) and R: (GCCTCAAGGGCACACATCGA) for Enterobacteriaceae and F: (AGCAGTACGGGAAATCTTCCA) and R: (CACCGCTACACATGGAG) for Lactobacillus (Thermo Fisher Scientific). 10-fold dilution standards were made from purified genomic DNA
from reference bacteria. Reactions were run at 95°C for 3’, followed by 40 cycles of 95°C for 15” and a 63°C (Bacteria) or 67°C (Enterobacteriaceae) for 60” using a Step One Plus Real-Time PCR instrument (Applied Biosystems, Foster City, CA).

**Enzyme-Linked Immunosorbent Assay.**

Mice in all four groups were sacrificed three hours after the last gavage on day 7. Large intestines were harvested and homogenized. The homogenates were analyzed for IL-22 (eBioscience) and IL-17 (R&D Systems) using their respective ELISAs per the manufacturer’s instructions. The cytokine levels were expressed per milligram of total protein in the homogenates.

**Western.**

Following sacrifice after the last gavage on day 7, large intestines were homogenized. Homogenates were analyzed by SDS-PAGE and were transferred to either PVDF or nitrocellulose membranes. The membrane was blocked for 1 hour at room temperature with 5% BSA in TBS-T (0.05% Tween 20 in TBS). Following this, the membrane was incubated with a desired antibody (e.g., anti-STAT3, Cell Signaling Technology, anti-pSTAT3, Cell Signaling Technology, or anti-β-actin, Cell Signaling Technology) overnight at 4°C. Membranes were washed with TBS-T and incubated with secondary antibody conjugated with horseradish peroxidase for one hour. After the incubation in the secondary antibody, the membrane was washed five times for five minutes in TBS-T and one time for 10 minutes in TBS. Following the final wash the membranes were probed using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer, Norwalk, CT). The membrane was visualized using a ChemiDoc System.

**Statistics.**

Comparisons within groups were analyzed using a two-Way ANOVA with a Tukey post-
hoc test. Analysis was done using GraphPad Prism software. A confidence level of p < 0.05 was considered statistically significant. Significance is represented as follows: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Results

We first examined the effect of rIL-22 treatment on the two most common assessments of UC severity in mouse models – weight loss and colon length. Figure 21A shows that DSS Ethanol treated mice repeatedly loose significantly more weight than DSS Vehicle mice, ~12.5% vs. ~6%, respectively. However, the average weight loss in DSS Ethanol mice treated with rIL-22 on days 5 and 6 was back to that of DSS Vehicle on day 7, ~6%. As we’ve previously observed, Figure 21B shows that our binge alcohol paradigm after DSS-induced colitis significantly decreases colon length. Treatment with rIL-22 in the DSS Ethanol +rIL-22 reverted colonic shortening back to levels of DSS Vehicle treated mice.
A.

B.

Colon Length (cm)

WT DSS Vehicle + PBS
WT DSS Ethanol + PBS
WT DSS Vehicle + rIL-22
WT DSS Ethanol + rIL-22
Figure 21. rIL-22 Treatment Prevented the Alcohol Induced Increase in Weight Loss and Colonic Shortening Following DSS-Induced Colitis. A. Percent weight change of animals was determined by the following equation: % weight change = (weight at day X-weight at day 0/weight at day 0)*100. Values are mean ± SEM 6-12 animals per group. By ANOVA on day 7 with Tukey post-hoc *p<0.05 WT DSS Ethanol vs. WT DSS Vehicle; **p<0.01 WT DSS Ethanol + rIL-22 vs. WT DSS Ethanol + PBS. B. Colon length measured in centimeters (cm) on day 7. Values are means ± SEM, n=6-12 animals/group. *p<0.05 WT DSS Ethanol vs. WT DSS Vehicle; ***p<0.01 WT DSS Ethanol + rIL-22 vs. WT DSS Ethanol + PBS by ANOVA with Tukey Post-hoc.

Alterations of the large intestine microbiome are commonly associated with severity of UC pathogenicity. Our lab has previously observed increases in Enterobacteriaceae, a potentially pathogenic family of bacteria, and decreases in Lactobacillus, a potentially beneficial family of bacteria, following DSS-induced colitis and alcohol. Therefore, we performed quantitative real-time PCR on 16S ribosomal RNA of Enterobacteriaceae and Lactobacillus on large intestine luminal content to determine whether treatment with rIL-22 could restore the microbial changes we observed in our model of binge alcohol and colitis. Copies of Total Bacteria within the large intestine luminal content via qRT-PCR analysis were used for normalization. Animals were housed in the same room and fed the same diet for a minimum of two weeks before beginning experiments to assimilate microbial exposure.

In line with our previous results, DSS Ethanol treated mice have a large increase in Enterobacteriaceae (~4 fold) on day 7 compared to DSS Vehicle treated mice. rIL-22 treatment not only reduced the Enterobacteriaceae copy number in the DSS Vehicle group, but also dramatically reduced Entero copy number in the DSS Ethanol group back to that of DSS Vehicle + PBS, Figure 22A. Copy number of Lactobacillus was decreased ~1 fold in DSS Ethanol mice compared to DSS Vehicle. Treatment with rIL-22 was able to partially restore Lactobacillus copy number, but it was not back to the level of DSS Vehicle, Figure 22B.
Figure 22. rIL-22 Prevents Overgrowth of Gram-negative Enterobacteriaceae and Partially Restores Lactobacillus Copy Numbers Following Alcohol and Colitis. Real-time PCR 16S rRNA sequencing of large intestine luminal content with primers specific for A. Enterobacteriaceae, B. Lactobacillus, and A-B. Total Bacteria.

IL-22 signals by binding to its cognate receptor on intestinal epithelial cells, which activates the transcription factor STAT3. To determine if IL-22 was mediating acute protective effects through STAT3, we administered IL-22 in STAT3 deficient mice and assessed weight loss, colonic shortening, and microbial dysbiosis. Figure 23A and B show that IL-22 administration in STAT3 -/- mice did not rescue weight loss or colonic shortening in DSS
Ethanol mice.
Figure 23. rIL-22 Treatment in STAT3−/− Could Not Prevent Alcohol Induced Increases in Weight Loss and Colonic Shortening Following DSS-Induced Colitis. A. Percent weight change of animals was determined by the following equation: % weight change = (weight at day X-weight at day 0/weight at day 0)*100. Values are mean ± SEM 6-12 animals per group. By ANOVA on day 7 with Tukey post-hoc *p<0.05 STAT3 −/− DSS Ethanol +rIL-22 against all other groups. B. Colon length measured in centimeters (cm) on day 7. Values are means ± SEM, n=6-12 animals/group.

qRT-PCR of Enterobacteriaceae in STAT3−/− mice shows that DSS Ethanol + PBS group of mice had dramatic increases in Enterobacteriaceae copy number compared to DSS Vehicle + PBS. Interestingly, unlike in wildtype mice where IL-22 treatment was able to mitigate the large increases in Enterobacteriaceae, STAT3−/− DSS Ethanol mice treated with IL-22 mice had even further increases in Enterobacteriaceae, Figure 24A.
Figure 24. rIL-22 Prevents Overgrowth of Gram-negative Enterobacteriaceae and Partially Restores Lactobacillus Copy Number Through STAT3 Following Alcohol and Colitis. Real-time PCR 16S rRNA sequencing of large intestine luminal content with primers specific for A. Enterobacteriaceae, B. Lactobacillus, and A-B. Total Bacteria.

Taken together, these data support our hypothesis that IL-22 promotes intestine health and mitigates microbial dysbiosis following binge alcohol and DSS colitis, however these IL-22 protective effects are dependent on STAT3 signaling. Although we observed beneficial effects with administration of recombinant IL-22, treatment of UC patients with a recombinant protein is not the most viable option due to potentially detrimental systemic side effects.

Probiotics are widely used and are available without a prescription. Therefore, we wanted to understand whether treatment with a bacteria commonly found in over the counter probiotics, Lactobacillus delbrueckii, could mediate protective effects in the context of binge
alcohol and UC. For these studies, we utilized our established model of 2% DSS in the drinking water of mice followed by a three-day binge alcohol paradigm or water. However, on day 4 mice were further subdivided into mice receiving 1X10^{11} CFUs of *Lactobacillus delbrueckii* on the evenings of day 4, 5, and 6. This resulted in four overall experimental groups – DSS Vehicle, DSS Ethanol, DSS Vehicle +*Lacto*, and DSS Ethanol +*Lacto*.

In line with ours and other’s previous reports, mice in both the DSS Vehicle and DSS Ethanol groups began losing weight on day 5 as is expected with the UC disease being induced by DSS. Furthermore, mice in the DSS Ethanol group lost significantly more weight compared to DSS alone, ~8% vs. ~4.5%, respectively; a consistent finding in our lab with the addition of ethanol to DSS treated mice. Interestingly, treatment with *Lactobacillus delbrueckii* not only mitigated weight loss (~2% body weight loss in DSS Vehicle + Lacto group vs. ~4.5% loss in DSS Vehicle mice), it also attenuated weight loss in the DSS Ethanol treated mice, which is demonstrated by the DSS Ethanol + Lacto group’s average weight loss returning to levels near that of DSS Vehicle on day 7 (Figure 25A). Colon length was also normalized with *Lactobacillus delbrueckii* treatment. Figure 25B shows that mice in the DSS Ethanol treatment group again experienced significant increases in colonic shortening compared to DSS Vehicle mice. However, when DSS Ethanol mice were given *Lacto*, DSS Ethanol + *Lacto* group, their colon length was back to that of DSS Vehicle.
Figure 25. Treatment with *Lactobacillus delbrueckii* Attenuated the Alcohol Induced Increases in Weight Loss and Colonic Shortening Following DSS Colitis. A. Percent weight change of animals was determined by the following equation: % weight change = (weight at day X-weight at day 0/weight at day 0)*100. Values are mean ± SEM n = 6-8 animals per group. *p<0.05 DSS Ethanol vs. DSS Vehicle by ANOVA on day 7 with Tukey post-hoc. B. Colon length measured in centimeters (cm) on day 7. Values are means ± SEM, n=6-8 animals/group. *p<0.05 DSS Ethanol compared to DSS Vehicle; ***p<0.01 DSS Vehicle + *Lacto* compared to DSS Ethanol; ****p<0.001 DSS Ethanol + Lacto compared to DSS Ethanol by ANOVA with Tukey Post-hoc.

Secondly, we sought to determine whether treatment with the probiotic, *Lactobacillus delbrueckii*, could also normalize aspects of the dysbiosis our lab has observed following DSS-induced colitis and binge alcohol consumption. We again performed quantitative real-time PCR on 16S ribosomal RNA of Gammaproteobacteria, *Enterobacteriaceae*, and *Lactobacillus* on large intestine luminal content with copies of Total Bacteria used for normalization. Our results showed a large 10 – fold increase in *Enterobacteriaceae* in DSS Ethanol mice compared to DSS Vehicle. However, as can be seen in Figure 26A, the large increase in *Enterobacteriaceae* seen in the DSS Ethanol group was impressively back to the levels of DSS Vehicle when DSS Ethanol mice were treated with Lacto. This accompanied a slight increase (0.5 – fold) of Gammaproteobacteria, the phylum in which *Enterobacteriaceae* belongs, in DSS Ethanol treated mice compared to DSS Vehicle, which also decreased back to levels lower than DSS Vehicle following treatment with *Lacto*, Figure 26B. Figure 26C shows that copies of *Lactobacillus* were decreased in DSS Ethanol compared to DSS Vehicle. Interestingly, when both groups were treated with *Lacto*, copies of *Lactobacillus* drastically increased in DSS Vehicle mice, but not DSS Ethanol. While the mechanism underlying this observation remains to be established, our data supports the hypothesis that treatment with Lactobacillus delbrueckii is able to modulate the alcohol-induced exacerbation of UC flare we see in our model of DSS-colitis and binge alcohol.
Figure 26. *Lactobacillus delbrueckii* Prevents Overgrowth of Gram-negative *Enterobacteriaceae*. Real-time PCR 16S rRNA sequencing of large intestine luminal content with primers specific for A. *Enterobacteriaceae* B. Gammaproteobacteria, C. *Lactobacillus*, and A-C. Total Bacteria.

Next, we wanted to elucidate the potential mechanism by which *Lactobacillus delbrueckii* provided these protective effects from UC flare and binge alcohol. As treatment with *Lactobacillus delbrueckii* has been shown to increase levels of IL-22, we assessed levels of large intestine IL-22. Consistent with our previous findings, levels of IL-22 in DSS Ethanol mice compared to DSS Vehicle were significantly decreased. In the DSS Ethanol + *Lacto* group, *Lacto* treatment was able to increase levels of IL-22, though not back to levels of DSS Vehicle.

Figure 27A. As the cytokines IL-22 and IL-17 are so closely related with IL-22 regarded as anti-inflammatory and IL-17 as pro-inflammatory, we also measured levels of colonic IL-17.
Treatment with *Lacto* in the DSS Ethanol group was able to reduce large intestine levels of IL-17 compared to DSS Vehicle, but not compared to DSS Ethanol, **Figure 27B**.

![Graph](image)

**Figure 27. Lactobacillus delbrueckii** Treatment Trended Towards an Increase in Large Intestine Levels of IL-22, but Did Not Decrease Levels of IL-17. A. Levels of total large intestine IL-22 quantified by ELISA. Values are mean ± SEM 6-8 animals per group. *p*<0.05 DSS Ethanol vs. DSS Vehicle by ANOVA with Tukey post-hoc. *p*<0.05 DSS Ethanol vs. DSS Ethanol + Lacto by student’s t-test. B. Levels of total large intestine IL-17 quantified by ELISA. Values are mean ± SEM 6-8 animals per group. *p*<0.05 DSS Ethanol + Lacto vs. DSS Vehicle by ANOVA with Tukey post-hoc.

One way IL-22 elicits its protective effects is through the phosphorylation and thus, activation of the transcription factor, STAT3. Phosphorylation of STAT3 allows for upregulation of genes involved in the production of AMPs and anti-inflammatory cytokines. Therefore, we measured levels of both STAT3 and pSTAT3 in large intestine homogenates following treatment with *Lactobacillus delbrueckii*. **Figure 28** shows that large intestine levels
of pSTAT3 in DSS Ethanol mice are decreased compared to that of DSS Vehicle. Interestingly, when DSS Ethanol mice were treated with Lacto, levels of pSTAT3 increased back to that of DSS Vehicle.

**Figure 28. Treatment with Lactobacillus delbrueckii Attenuates Decreased Levels of pSTAT3 in DSS Ethanol Mice.** Protein isolated from total large intestine tissue was probed for STAT3 and pSTAT3 (Y705) by Western blot. Densiometric analysis was performed to express the ratio of pSTAT3/β-actin.

Finally, we wanted to answer the question of whether the beneficial effects we observed following treatment with *Lactobacillus delbrueckii* was due to the bacteria itself or a potential factor released by the bacteria. As can be seen in **Figure 29A and B,** in order to reduce weight loss and colonic shortening back to the level of DSS Vehicle treated mice, DSS Ethanol treated mice must receive whole Lacto bacteria. Treatment with Lacto supernatant alone, DSS Ethanol
+ *Lacto* Sup group, resulted in weight loss and colonic shortening comparable to that of DSS Ethanol mice that were not treated with *Lacto*.
Figure 29. *Lactobacillus delbrueckii* Bacteria are Required to Attenuate the Alcohol Induced Increases in Weight Loss and Colonic Shortening Following DSS Colitis. A. Percent weight change of animals was determined by the following equation: % weight change = (weight at day X - weight at day 0 / weight at day 0) * 100. Values are mean ± SEM n = 5 animals per group. **p<0.01 DSS Ethanol vs. DSS Vehicle by; **p<0.01 DSS Ethanol + Lacto Sup. vs DSS Vehicle by ANOVA on day 7 with Tukey post-hoc. B. Colon length measured in centimeters (cm) on day 7 with Tukey post-hoc. Values are means ± SEM, n=5 animals/group. *p<0.05 DSS Ethanol compared to DSS Vehicle; **p<0.01 DSS Ethanol + Lacto Sup compared to DSS Vehicle; ns= not significant DSS Ethanol + Lacto Bacteria vs DSS Vehicle by ANOVA with Tukey Post-hoc.

**Summary**

Here, we demonstrated that the alcohol induced exacerbation of UC symptoms including increased weight loss, colonic shortening, and large intestine *Enterobacteriaceae* copy number can be attenuated by exogenous administration of recombinant IL-22. Our findings further demonstrate that IL-22-mediated protection requires STAT3 signaling in intestinal epithelial...
cells, as STAT3−/− knockout mice did not benefit from IL-22 treatment following DSS-induced colitis and alcohol.

Treatment with the probiotic, *Lactobacillus delbrueckii*, was also able to mediate protection against exacerbated UC symptoms of weight loss and colonic shortening following binge alcohol consumption. Furthermore, *Lacto* treatment was able to reduce the large increases in the pathogenic bacteria, *Enterobacteriaceae*, that we see following DSS-colitis and binge alcohol back to that of non-alcohol treated mice. As treatment with *Lacto* was also able to increase levels of IL-22, which resulted in increased levels of pSTAT3, our results highlight a potential mechanism by which this probiotic elicited protective effects against alcohol-induced worsening of UC flare. Therefore, *Lactobacillus delbrueckii* treatment could potentially be a therapeutic option for UC patients experiencing symptom exacerbation and/or prolonged flare due to alcohol use by tipping the scales into a period of remission.
CHAPTER SIX

BINGE ALCOHOL FOLLOWING DSS-INDUCED COLITIS INCREASES SUSCEPTIBILITY TO INFECTION

Abstract

Ulcerative colitis (UC) is a life-long disease with periods of remission and disease flares characterized by abdominal pain, increased weight loss, intestinal inflammation, and rectal bleeding. In addition to treatment with medication, UC patients are commonly warned against alcohol use. One study found IBD patients self-reported a worsening of GI symptoms following alcohol consumption. With our mouse model of binge alcohol exposure following induced colitis, we found increased weight loss, colon shortening, and large intestine inflammation in mice exposed to alcohol. We sought to elucidate whether alcohol consumption not only perpetuated an UC flare, but also whether alcohol increased susceptibility to the enteropathogen C. rodentium. Male mice received DSS for 5 days. On days 5, 6, and 7 mice were gavaged with alcohol (~3g/kg) or water. Three hours after the last gavage on day 7, mice were orally administered C. rodentium at 1 X10^5 CFUs. Body weight and mortality were monitored. On day 11, mice were euthanized and colons harvested to measure length, inflammatory markers such as IL-18, IL-1β, IL-6, TNFα, and KC proteins levels via ELISA, and the tight junction protein expression. Colon sections were stained by H&E and alcian blue. C. rodentium infection in mice following DSS and alcohol treatment resulted in increased weight loss compared to those receiving DSS alone and C. rodentium. We observed a 50% reduction in survival in DSS alcohol treated mice following C. rodentium infection compared to 100% survival with DSS and C.
rodentium. This accompanied a significant decrease in colon length in DSS alcohol treated mice following C. rodentium infection compared to DSS and C. rodentium. Mice infected with C. rodentium following DSS and alcohol had significant decreases in claudin 8 and occludin compared to all groups. H&E staining revealed prominent colonic damage and inflammatory infiltrate in DSS alcohol plus C. rodentium infected mice. Histopathology was scored in a blinded fashion, and we found significant increases in histopathology scores in DSS alcohol and C. rodentium treated mice compared to DSS and C. rodentium. In the DSS ethanol and C. rodentium group, 8/12 mice showed a decrease in the mucus layer along with a decrease in goblet cells. Mice in the DSS Ethanol + C. rod group experienced significantly increased levels of IL-18 and IL-1β in colonic homogenates compared to DSS Vehicle + C. rod. IL-6 was increased in both the DSS Ethanol + C. rod and DSS Vehicle + C. rod compared to mice treated with DSS alone. Interestingly, levels of TNFα and KC were not further increased following C. rod infection after DSS Ethanol treatment. Utilizing luciferase expressing C. rod, we were also able to show that mice infected with C. rodentium following DSS and alcohol had increased colonization by C. rodentium on day 11 compared to DSS Vehicle + C. rod treated mice. Along with our previous findings, these data suggest alcohol increases susceptibility to enteropathogens in mice with DSS-induced colitis.

Introduction

Under normal homeostatic conditions, the large intestine employs a whole host of defenses against invading pathogenic bacteria. Firstly, the mucus layer serves to protect barrier integrity by limiting the amount of interaction between luminal contents and intestinal epithelial cells. Irregular mucin profiles of the most prevalent mucin in the colon, mucin-2, have been
demonstrated in disease states of IBD. Furthermore, decreased mucin-2 expression correlated with increased ability of pathogens to cross the intestinal barrier.

Secondly, proper intestinal function requires the formation and maintenance of a selective barrier formed by tight junction proteins of intestinal epithelial cells, which retain commensal microbes to the lumen while allowing passage of critical nutrients. Literature from other laboratories has shown significant disruptions in claudin-2, claudin-4, and occludin expression in colons of patients with UC.

Thirdly, proper levels of inflammation are the body’s response to harmful stimuli, such as invading pathogens. Pattern-recognition receptors (PRRs) on macrophages will recognize antigens on microbes which initiates a coordinated inflammatory cascade involving the release of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα. Activated macrophages will also release pro-inflammatory chemokines, such as KC, which signal the recruitment of neutrophils, NK cells, and other pro-inflammatory lymphocytes. In a healthy individual, this inflammatory cascade is coordinated in such a way to maintain tolerance to the body’s commensal microbes, but eliminate pathogenic bacteria. In the context of UC, tolerance towards the resident microbiome is broken, resulting in an over activation of inflammatory pathways. Consequently, these consistently high levels of intestinal inflammation produce severe tissue damage, thus compromising the integrity of the intestinal barrier leaving it susceptible to invading pathogens.

Therefore, in addition to the many other co-morbidities associated with UC, patients experience higher rates of intestinal infections. Further compounding the problem, treatment of UC-associated intestinal inflammation with the above listed anti-inflammatory treatments results in overall immunosuppression, potentially allowing the invasion of pathogenic bacteria that would have normally been defended against. Furthermore, changes in bacterial populations from
broad-spectrum antibiotic treatment could open specific niches more favorable to invading pathogens, which then gives rise to the increased susceptibility to bacterial infections seen in IBD/UC patients. Thus by simply treating the symptoms of UC (inflammation and dysbiosis), we could actually be feeding the cycle of remission to flare to remission. These opposing ideas make critical the need for further research into what could be leading to UC patients experiencing higher rates of infection. Knowing that alcohol alone can increase propensity to infection, we sought to understand whether alcohol consumption in a model of UC plays a role in increased rates of infection seen in UC patients.

Our results demonstrate that mice receiving *C. rodentium* following alcohol and DSS-induced colitis had decreased survival and increased weight loss, colon shortening, histopathology scores, a decreased colonic mucosal layer and goblet cell number, decreased tight junction protein expression, increased inflammation, and increased colonization by *C. rodentium*. These findings highlight alcohol’s ability to potentiate susceptibility to infection in UC, and show the dramatic increase in UC symptoms following a bacterial infection when intestinal barrier defenses are further compromised by the consumption of alcohol.

**Materials and Methods**

**Induction of DSS Colitis and Binge Alcohol.**

Male 8-9 week old (~23-25g body weight) C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). Briefly, as can be seen in Figure 30, mice were randomly separated into four experimental groups: Sham Vehicle, Sham Ethanol, DSS Vehicle, and DSS Ethanol. DSS treated mice received 2% (wt/vol) DSS (40,000 kDa; MP Biomedicals), *ad libitum*, in their drinking water for five days. Mice in the Sham group received water only for 5 days acting as a control. On day 5, DSS was discontinued and replaced with normal drinking
water in both the DSS and Sham/control groups. On day 5, mice in both the DSS and
Sham/control group were further subdivided into two subgroups: mice gavaged with alcohol
(~3g/kg) or mice gavaged with water on days 5, 6, and 7 to mimic a binge alcohol abuse pattern.
The amount of DSS water drank per animal was recorded and no differences in intake between
groups were observed. Mice were weighed every day for the determination of percent weight
change. This was calculated as: % weight change = (weight at day X- weight at day 0/weight at
day 0) X 100. Animals were monitored clinically for rectal bleeding, diarrhea, and general signs
of morbidity, including hunched posture and failure to groom.

**Citrobacter rodentium (C. rod) Infection.**

Mice were subjected to the DSS-induced colitis and binge alcohol paradigm as described
above. However, after the last gavage on day 7, mice were further divided into two subgroups:
mice gavaged with 1 X 10^5 CFUs of C. rod suspended in 300 mL of PBS or 300 mL of PBS alone. This resulted in four experimental groups: DSS Vehicle, DSS Ethanol, DSS Vehicle + C.
rod, and DSS Ethanol + C. rod.

For C. rod colonization experiments, a bioluminescent strain of C. rodentium was used.
This strain was gifted to us by Dr. Sara Jones of Loyola University Chicago who had the
permission of Dr. Gad Frankel (Imperial College London), whose lab created this strain. In short,
the luxCDABE operon was introduced into the C. rodentium bacterial strain. The luxCDABE
operon encodes for luciferase and the enzymes required for aldehyde substrate recycling.
Bioluminescent colonies were plated to purity as single colonies and lux+ phenotype confirmed
by luminometry prior to storage under glycerol at −80°C. Mice were separated into two groups:
, DSS Vehicle + C. rod lux+, and DSS Ethanol + C. rod lux+, both of which received 1 X 10^5
CFUs of C. rod lux+ suspended in 300 mL of PBS three hours post the last gavage of either
alcohol or water on day 7. On day 11, mice were gavaged with 2mg/200uL of the luciferase substrate, Vivoglo luciferin (Promega). Mice were euthanized 2 hours post luciferin gavage and large intestines were harvested to directly assess colonization utilizing an IVIS \textit{in vivo} imaging system (available in Loyola’s Imaging core), which captures images of photon emission from the bioluminescent \textit{C. rod}.

The experiments described here were carried out in adherence with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and are approved by the Institutional Animal Care and Use Committee, Loyola University Chicago Health Sciences Division, Maywood IL.

\textbf{Tissue Staining.}

For hematoxylin and eosin (H&E) staining, 2cm of colon tissue closest to the rectum was taken from each mouse and saved in 10% formalin. Tissue was fixed with 10% phosphate buffered formalin, paraffin embedded, sectioned at 5 μm, and stained with hematoxylin and eosin or alcian blue, which stains mucin, by AML laboratories (Saint Augustine, Florida). Images were taken on an Olympus BX43 Microscope using an Olympus DP26 camera.

\textbf{Histopathology Scoring.}

H&E stained sections were analyzed and scored in a blinded manner by Dr. Xianzhong Ding. Dr. Ding is an Associate Professor of Pathology at Loyola University Chicago and member of my Dissertation Committee. Scoring was based on a modified 0-4 point scale examining exudate, epithelial damage, polymorphonuclear leukocyte invasion, and submucosal edema20. The values from each of the 4 categories were added to produce a combined histopathology score for each animal.
Colon Length.

Immediately following euthanasia, colons were excised and length measured.

Real-time PCR Gene Analysis.

Anti-microbial peptide transcript levels were measured using TaqMan Gene Expression Assay. Briefly, RNA was isolated from intestinal homegenates using a Qiagen RNEasy Kit per the manufacturer’s instructions (Qiagen, Hilden, Germany). Reverse transcription was performed using a High Capacity Reverse Transcription Kit (Applied Biosystems), and real-time PCR were performed per manufacturer’s instructions. Primers for Claudin 2, Claudin 4, Claudin 8, Occludin, and GAPDH were obtained from Applied Biosystems.

Enzyme-Linked Immunosorbent Assay.

Mice in all four groups were sacrificed on day 11, as seen in Figure 30. Large intestines were harvested and homogenized. The homogenates were analyzed for IL-18 (eBioscience), IL-1β (R&D Systems), IL-6 (R&D Systems), TNFα (eBioscience), and KC (BD Biosciences) using their respective ELISAs per the manufacturer’s instructions. The cytokine levels were expressed per milligram of total protein in the homogenates.

Statistics.

Comparisons within groups were analyzed using a one-Way ANOVA with a Tukey post-hoc test. Analysis was done using GraphPad Prism software. A confidence level of p < 0.05 was considered statistically significant. Significance is represented throughout the manuscript as follows: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Results

UC patients are at higher risk of developing bacterial infections28,29. To understand whether consumption of alcohol not only impacts UC patient’s increased susceptibility to
infection but also increases severity of symptoms related to bacterial infection, we utilized our model of DSS-induced colitis and binge alcohol, as described above, along with a single inoculation of *C. rod*, a well-known Gram negative enteropathogen associated with colonic infection, at 1 \times 10^5 \text{ CFUs} three hours after the last gavage on day 7 (Figure 30).

![Figure 30](image.png)

**Figure 30. Murine Model of DSS-Induced Colitis, Ethanol, and *C. rodentium* Infection.** A 2% DSS concentration was administered ad libitum in drinking water for 5 days to mimic symptoms of UC. On day 5, DSS was discontinued to allow entrance into UC remission. A binge alcohol paradigm was employed where mice were gavaged with alcohol or water on days 5, 6, and 7. Mice were further subdivided and were gavaged with either 1 \times 10^5 \text{ CFUs} *C. rodentium* or water 3 hours post last gavage on day 7. Mice were euthanized on day 11.

As can be seen in Figure 31, the percent survival of DSS Ethanol treated mice following *C. rod* infection fell to 50% by day 11 compared to 100% survival in the DSS Vehicle group with *C. rod* infection. Interestingly, DSS Ethanol mice with no *C. rod* infection also experienced a 20% reduction in survival compared to the 100% survival in the DSS Vehicle group with and without *C. rod* infection.
Figure 31. Ethanol Decreased % Survival Following C. rodentium Infection. n=7-8 animals/group.

Weight loss and gain was also monitored as described previously by percent change from day 0 up till day 11 as only 50% of the mice in the DSS Ethanol + C. rod infection group survived till day 11. By day 11 following C. rod infection, mice in the DSS Ethanol group experienced a ~27% decrease from their original body weight compared to ~22% in the DSS Vehicle group (Figure 32A) giving evidence to our hypothesis of alcohol not only increasing susceptibility to infection with UC, but also increasing severity of symptoms associated with UC and infection. Increased weight loss accompanied increases in colonic shortening in the DSS Ethanol + C. rod group compared to mice in the DSS Vehicle + C. rod group, Figure 32B.
Figure 32. Alcohol Consumption Increases Weight Loss and Colonic Shortening Following *C. rodentium* Infection in DSS-Induced Colitis. A. Alcohol consumption increases weight loss following *C. rodentium* infection in DSS-induced colitis. Values are calculated as average % weight change, *p*<0.05 DSS Vehicle + *C. rod* compared to DSS Vehicle; **p**<0.01 DSS Ethanol + *C. rod* compared to DSS Vehicle by Two-way ANOVA, n=7-8 animals/group. B. Increased colonic shortening with *C. rodentium* infection after alcohol consumption and DSS-induced colitis. Values are means ± SEM, n=7-8 animals/group. *p*<0.05 DSS Ethanol compared to DSS Vehicle; ****p**<0.0001 DSS Ethanol + *C. rod* compared to DSS Vehicle and DSS Vehicle + *C. rod* ANOVA.

Again, to understand differences in histopathology following infection with *C. rod*, sections of colon were taken closest to the rectum, stained via H&E, blinded, and scored by a pathologist. Figure 33A shows gross differences in large intestine morphology after DSS Ethanol + *C. rod* treatment compared to all other groups. As in Figure 9B, inflammatory infiltrate and epithelial damage were assessed and were severely increased in the DSS Ethanol + *C. rod* mice compared to that of the DSS Vehicle + *C. rod* group. The combined histopathology
scores in DSS Ethanol + *C. rod* treated mice were significantly increased compared to mice in the DSS Vehicle + *C. rod* group, **Figure 33B.**
Increased colonic damage and inflammatory infiltrate with *C. rodentium* infection after alcohol consumption and DSS-induced colitis. Representative H&E images, n=6-12 animals/group. B. Combined Histopathology Score following blinded histological scoring as described in detail in Methods section above. Values are mean ± SEM, n=6-12 animals/group. **p<0.01 DSS Ethanol + *C. rod* compared to DSS Vehicle; *p<0.05 DSS Ethanol + *C. rod* compared to DSS Vehicle + *C. rod* and DSS Ethanol by ANOVA.

To further assess colonic barrier breakdown following *C. rod* infection, we performed an Alcian blue stain, which stains the glycoproteins found in the mucosal layer lining intestinal epithelial cells and in large intestine goblet cells. Eight out of the twelve mice in the DSS Ethanol + *C. rod* group showed a decrease in the mucus layer along with a decrease in goblet cells as can be seen in representative images in Figure 34.
Proper intestinal barrier function is dependent on the formation and integrity of tight junction protein complexes adhering adjacent intestinal epithelial cells. **Figure 35C and 35D** show that *C. rod* infection results in a significant decrease in expression of both Claudin 8 and Occludin in the large intestine of DSS Ethanol + *C. rod* mice, respectively. Expression of Claudin 2 (**Figure 35A**) and Claudin 4 (**Figure 35B**) were not significantly altered in the DSS Ethanol treated mice following infection with the colonic pathogen, *C. rodentium*. 

**Figure 34.** Decreased Large Intestine Mucosal Lining and Goblet Cell Number with *C. rodentium* Infection After Alcohol Consumption and DSS-Induced Colitis. Representative Alcian blue images, n=6-12 animals/group.
As we did previously, seen in Figure 11, we assessed colonic inflammation under the hypothesis that *C. rod* infection would further increase inflammation in mice receiving DSS Ethanol treatment, which could perpetuate increased colonization of *C. rod*. We found that mice in the DSS Ethanol + *C. rod* group had increased levels of IL-18 (Figure 36A) and IL-1β (Figure 36B) compared to DSS Vehicle + *C. rod*. Furthermore, the pro-inflammatory cytokine IL-6 was increased in both the DSS Ethanol + *C. rod* and DSS Vehicle + *C. rod* compared to mice treated with DSS alone (Figure 36C). However, both TNFα and KC were not further increased following *C. rod* infection after DSS Ethanol treatment (Figure 36D and 36E).
**Figure 36. C. rodentium Further Increases Colonic Inflammation After DSS-Induced Colitis and Ethanol Treatment.** Colons were harvested, homogenized, and processed on day 11 for the analysis of inflammatory mediators using respective ELISAs. A. IL-18 *p<0.05 DSS Ethanol + C. rod compared to DSS Vehicle + C. rod and DSS Vehicle by ANOVA, B. IL-1β *p<0.05; **p<0.01; ***p<0.001 DSS Ethanol + C. rod compared to all other groups by ANOVA, C. IL-6 *p<0.05 DSS Ethanol + C. rod compared to DSS Vehicle and DSS Vehicle + C. rod compared to DSS Vehicle by ANOVA, TNFα, E. KC by ELISA. Values are mean ± SEM 7-8 animals per group.

Finally, to assess whether mice in the DSS Ethanol group were truly more susceptible to infection, we utilized a bioluminescent strain of C. rod gifted to us by Dr. Sara Jones to image C. rod colonization of the large intestine following DSS-induced colitis and binge alcohol. The luxCDABE operon was introduced into the C. rod strain and bioluminescent colonies (lux+) were plated to purity and utilized for these experiments. As can be seen in **Figure 37**, mice in
the DSS Ethanol + *C. rod* lux+ group have higher levels of photon emission (as seen by increased red and blue areas) in their intestines indicative of increased *C. rod* colonization compared to DSS Vehicle + *C. rod* lux+.

**Figure 37. Increased *C. rodentium* Colonization in DSS Ethanol Mice Compared to DSS Vehicle.**

**Summary**

Research has shown that IBD patients and alcoholic patients carry an intestinal bacterial dysbiosis\(^{50,83}\). A dysbiosis is believed to provide pathogens an opportunity to colonize and proliferate\(^{158}\). Indeed, studies have shown IBD patients and alcoholics are at risk for increased infections\(^{159,160}\). In a retrospective analysis of patients with a documented history of alcohol use and IBD, our lab found that these patients had increased intestinal infections. With our low inoculate of *C. rodentium* in our murine model of binge alcohol and DSS-induced colitis, we were able to mimic these patient’s results as DSS Ethanol treated mice had increased
susceptibility and colonization of *C. rodentium*. We recognize the burden of utilizing the model pathogen, *C. rodentium*, which itself is used as a model of IBD, on top of our binge alcohol and DSS-induced colitis model. Yet, our adaptation of using a much lower inoculate, 1 X 10⁵ CFUs vs. 1 X 10¹¹ CFUs to induce true IBD symptoms, allowed us to shed light on the increased propensity for IBD patients who drink alcohol to acquire intestinal infections¹⁶¹.
CHAPTER SEVEN

DISCUSSION

New Contributions to the Alcohol and Ulcerative Colitis Field

The overarching goal of this work was to expand our understanding of how binge alcohol drinking could potentially worsen GI symptoms of UC and to identify potential treatment regimens to improve lives of UC patients. While a good amount of work has been and still is dedicated to elucidating a cure for UC, it is imperative that research continues in attempt to not only understand the triggers of flare, but also maintenance therapies to keep patients in remission and out of active UC flare. A report from the U.S. Department of Health and Human Services at the Centers for Disease Control and Prevention stated that the peak age at which patients are diagnosed with IBD is between 20-40\textsuperscript{1}. Interestingly, a study conducted by Naimi et al. found this is also the age range where an estimated 70% of binge alcohol drinking episodes occur\textsuperscript{15}. These two independent observations highlight the need to investigate a potential correlation between binge alcohol drinking and UC diagnoses. However, contrasting evidence as to whether drinking alcohol worsens UC symptoms in patient populations made it necessary for our lab to generate a mouse model of UC and binge alcohol drinking.

The results presented here demonstrate that binge alcohol drinking can exacerbate an UC flare period in a mouse model of UC shown by increased weight loss, colonic shortening, histopathology and clinical scores, inflammation, microbial dysbiosis, failure to release a critical cytokine involved in entrance into remission, IL-22, and susceptibility to infection. Administration of recombinant IL-22 and the probiotic, \textit{Lactobacillus delbrueckii}, were effective
in restoring these exacerbated symptoms in a STAT3-dependent fashion. As a whole, these findings provide a new insight into cellular and molecular mechanisms that may contribute to intestine barrier disruption following an UC flare period in the presence of intoxication, and this may have implication in other forms of autoimmune conditions that acutely affect intestinal physiology.

**Generating a Murine Model of UC and Alcohol**

One limitation of UC research is that no one murine model exactly replicates UC, as UC pathology is so multi-factorial. Therefore, researchers are restricted to mimicking colitis symptoms in murine model systems with various chemicals such as DSS, of 2,4,6-trinitrobenzenesulfonic acid (TNBS), or oxazolone. These chemicals damage the integrity of the large intestine triggering innate and adaptive inflammatory cascades and intestinal dysbiosis, which presents UC-like symptoms. Whether the inflammation or the dysbiosis is the initiating factor in inducing the other is still unknown and a matter of debate amongst UC researchers. Our laboratory choose to utilize DSS in generating our model due to its reproducibility in initiating UC symptoms and ease use. Anywhere from a 1-5% concentration of DSS is used in the field of UC research. Therefore, when generating our model, we first profiled 2, 3, and 4% concentrations in order to understand not only what works in our hands and laboratory environment, but also to whether DSS produced the expected UC symptoms. The exact mechanism behind which DSS damages the intestinal barrier is still unknown. However, DSS-induced intestinal damage is likely through a combination of disturbance in the metabolism of phospholipids, which are major cellular constituents required for the assembly of biological membranes and loss of tight junction protein ZO-1, which could facilitate in increasing intestinal...
permeability. We found that all three concentrations of DSS we utilized, indeed, were able to induce UC-like symptoms in treated mice.

The NIAAA defines binge drinking as a pattern of drinking that brings blood alcohol concentration (BAC) levels to 0.08 g/dL in a single dose. For the past 15 years our lab has had a murine model of alcohol and burn injury, which utilizes a single dose of alcohol at ~3g/kg per mouse. Therefore, we used this alcohol dose when generating our model of UC and binge alcohol. We tested two binge alcohol paradigms, a one-day binge on the last day as seen in Figure 4 and a three-day binge as seen in Figure 6. Our results demonstrated that a 2% DSS concentration followed by a three-day binge alcohol paradigm was the best experimental model to begin to elucidate how alcohol could be perpetuating an UC flare. We were able to show that DSS Ethanol treated mice had exacerbated symptoms of UC as shown by increases in weight loss, colon shortening, more profound histopathology and clinical scores, and increased intestinal inflammation, all of which are standard assessment of UC severity in mouse models. To our knowledge, this is the first time a murine model of UC and alcohol has been developed to allow a better understanding of how drinking alcohol could affect a patient with UC.

However, two questions remain: 1) Whether alcohol can trigger an UC flare? and 2) Whether drinking alcohol predisposes one to a UC diagnosis?

**Alcohol Use as a Trigger and/or Predisposition for UC**

The results of our work demonstrate alcohol’s ability to worsen an induced flare period (i.e. 5 days of DSS). Once DSS was removed on day 5, DSS Vehicle treated mice showed signs of entrance into remission as seen by less severe histopathology and clinical scores, decreased intestinal inflammation, and increased protein levels of IL-22 compared to DSS Ethanol treated
mice. We recognize the limitation in our alcohol and colitis model that a person experiencing an UC flare might not participate in an alcohol binge.

Therefore, future work will focus on inducing an UC flare with DSS in the drinking water for 5 days as per our model in Figure 6, but instead of an immediate binge of alcohol, mice will be allowed to recover for one week to model entrance into UC remission. Then we will implement our three-day binge alcohol paradigm to assess whether a binge of alcohol during a period of UC remission can trigger an UC flare. Preliminary data from our laboratory utilizing this model does indeed show that an alcohol binge can induce UC flare symptoms even during an UC remission period.

To answer the question of whether alcohol could predispose one to UC, we will utilize an IL-10 deficient mouse. IL-10 is an immunoregulatory cytokine essential in the maintenance of intestinal homeostasis. A wide range of immune cells secrete IL-10 such as macrophages, dendritic cells, and T cells, which suppresses effector functions of Th1/Th17 cells as well as NK cells and macrophages, thereby modulating the cellular immune response\textsuperscript{162-164}. Interestingly, a genetically engineered IL-10–deficient mouse has been extensively used to dissect IBD etiology. IL-10 knockout mice were first generated in 1993 by Kühn et al.\textsuperscript{165}. The generation was performed by use of targeted mutation disrupting the IL-10 gene by replacing a 500 base pair fragment of exon 1 with a termination codon and a neo expression cassette and by introducing a termination codon into exon 3 (Il10tm1Cgn, IL10−/−). IL-10 deficiency produces discontinuous and transmural inflammatory lesions characterized by inflammatory cell infiltrates into the lamina propria and submucosa, epithelial hyperplasia, mucin depletion, crypt abscesses, ulcers, and thickening of the intestinal wall\textsuperscript{166,167}, all of which are symptoms of human UC. The onset of gut inflammation in IL-10 knockout mice occurs spontaneously after weaning\textsuperscript{168}.
To answer the question of whether alcohol consumption increases risk of UC diagnosis, we hope to utilize IL-10 knockout mice and employ a binge alcohol paradigm before UC spontaneously sets in. If a binge of alcohol produced UC symptoms at an earlier time point or in an exacerbated passion, this would provide evidence for alcohol use directly influencing UC risk. Thus, future work will be dedicated in designing a binge alcohol paradigm in IL-10 knockout mice.

**Interleukin-22 in UC Remission**

One potential pathway that upon its activation could act in the amelioration of DSS-induced colitis is the aryl hydrocarbon receptor (AhR) pathway. Studies have shown that activation of the AhR pathway not only through chemical activation via TCDD (2,3,7,8-tetrachlorodibenzo-pdioxin) but also by specific probiotics is able to inhibit DSS-induced colitis\(^\text{132,169}\). Additionally, cytokines known to be induced upon AhR activation, specifically IL-22, are known to be upregulated during remission periods of UC\(^\text{98,143}\).

IL-22 remains one of the most intriguing cytokines due to its ability to elicit completely different responses based on the microenvironment. In the context of the intestines, the presence of IL-22 appears to be beneficial under most circumstances including inflammatory bowel disease, graft-versus-host disease, and many types of bacterial infection\(^\text{102,153,170-173}\). Interestingly, certain bacterial infections, such as in mouse models using *T. gondii*, cause IL-22 to be pro-inflammatory\(^\text{174,175}\). While the reasons behind these differential responses of IL-22 remain unknown, it illuminates the importance of understanding the role of IL-22 under different conditions. In addition, current clinical trials using Fc-fusion IL-22 administration for treatment of patients with graft-versus-host disease have shown promising preliminary results, indicating that IL-22 treatment may be efficacious in a clinical setting\(^\text{176}\).
The increased weight loss, colonic shortening, more profound histopathology and clinical scores, and increased intestinal inflammation outlined in Chapter 3 clearly demonstrate alcohol’s role in increasing symptoms of UC flare. Combined with the knowledge that IL-22 is a critical cytokine for entrance into UC remission, we examined whether alcohol was affecting IL-22 levels and thus perpetuating UC flare. Our results show that mice allowed to recover for 3 days (DSS Vehicle group) experienced increased levels of IL-22 in large intestine homogenates. In mouse models of colitis, the innate immune response in the colon includes recruited macrophages and neutrophils, which appear to have both pro-inflammatory and anti-inflammatory roles in colitis\textsuperscript{177}. Specifically, aberrant control of these infiltrating neutrophils can result in tissue damage in the colon caused by abundant reactive oxygen species\textsuperscript{178-180}. However, recent studies demonstrated that neutrophils can play a protective role in the host response in acute models of colitis by producing IL-22\textsuperscript{181,182}. Hence, we examined whether the spike in IL-22 levels in DSS Vehicle mice was due to infiltrating neutrophils, and conversely whether the diminished IL-22 response in DSS Ethanol mice was a consequence of abnormal control of IL-22+ producing neutrophils. We found that both large intestines of DSS Vehicle and DSS Ethanol mice had increased percentage of neutrophils. In contrast to Zindl et al.’s results, these isolated neutrophils were not producing IL-22 in our model of DSS-induced colitis and alcohol.

However, examination IL-22+ γδ T cells revealed that DSS Vehicle treated mice had a significantly increased percentage of IL-22+ γδ T cells, but this response was abolished in DSS Ethanol treated mice. This balance between decreased percentages of IL-22+ neutrophils and simultaneous decrease in percentages of IL-22+ γδ T cell in DSS Ethanol mice gives evidence to how alcohol could be damaging the critical response IL-22 needed to restore the integrity of the...
intestine following mucosal injury and enter into UC remission. Therefore, increasing levels of large intestine IL-22 in the context of binge alcohol consumption and UC could act a potential therapeutic target to improve lives of UC patients.

In the intestine IL-22 can stimulate proliferation, mucous protection, and AMP secretion, which could drive entrance into the remission period from a UC flare\textsuperscript{99,104,111,182,183}. The present study provides a mechanistic role for IL-22 mediated protection against alcohol induced UC flare through STAT3. A recent report supporting this mechanism from Hanash \textit{et al.} showed that IL-22 specifically signals through STAT3 in stem cells within the crypts of both small and large intestines to promote barrier regeneration in a murine model of graft versus host disease\textsuperscript{184}. While IL-22 has been shown in other models to signal through other STATs or MAPK/ERK pathways\textsuperscript{185-188}, we found epithelial cell STAT3 to be necessary for IL-22 mediated protection against alcohol-induced exacerbation of DSS-induced colitis.

Although this is an exciting finding for the field of alcohol and colitis research, translating this treatment of an exogenous protein into UC patients could possibly have systemic side-effects. Therefore, we chose to examine probiotics as a potential therapeutic option has they are readily available without a prescription.

**Probiotic Intervention as a Potential Therapeutic Option**

Interestingly, we also found that treatment with the probiotic, \textit{Lactobacillus delbrueckii}, attenuated the increased severity of UC symptoms we observe after DSS-induced colitis and alcohol. \textit{Lacto} treatment was able to upregulate activated colonic STAT3 following colitis and alcohol back to that of DSS Vehicle treated mice providing mechanistic evidence for how \textit{Lactobacillus delbrueckii} mediates its protective effects.
We acknowledge that this work does not currently have any functional read outs of the benefits of *Lacto* treatment. As activated STAT3 is known to increase intestinal epithelial cell proliferation, increase AMPs, and increase immunomodulatory cytokines such as IL-10, we expect *Lacto* treated mice to also have increases in these downstream targets of activated STAT3. Additionally, we expect *Lacto* treatment to increase percentages of IL-22+ lamina propria immune cells in DSS Ethanol treated mice. Our findings in Chapter 5 not only gives evidence to γδ Tcells as the potential source of IL-22 in DSS Vehicle treated mice, which models entrance into UC remission, but also points to an inability of γδ T cells to mount the proper IL-22 response in the presence of alcohol. Finally, we expect FACS analysis of large intestine lamina propria immune cells following *Lacto* treatment to increase the percentage of IL-22+ γδ T cells in DSS Ethanol mice.

**Increased Susceptibility to Infection**

Besides symptoms associated with UC itself, UC patients have a higher propensity to other co-morbidities such as infection. Patients with UC are hospitalized commonly with worsening diarrhea, which in many instances is attributable to progression of the underlying IBD, but it might also be the result of infection with enteric organisms or opportunistic agents such as cytomegalovirus and *C. difficile*. Thus, prompt diagnosis and treatment of infection become paramount. Previous studies have reported that 5%–19% of patients admitted for relapsing IBD are positive for *C. difficile*. Rodemann et al. performed a retrospective cohort study by using inpatient electronic medical records to determine the incidence of *C. difficile* associated diarrhea in hospitalized IBD patients and found the incidence in IBD is higher than in the non-IBD patients. IBD and UC patients in particular had a higher risk for *C. difficile* associated diarrhea. These data combined with our laboratory finding strong evidence of
alcohol’s role in IBD pathogenicity\textsuperscript{192}, we sought to answer the question of whether consumption of alcohol not only impacts UC patient’s increased susceptibility to infection but also increases severity of symptoms related to bacterial infection.

Hence, we adapted our model of UC and binge alcohol to include a low inoculate of \textit{C. rodentium} in order to understand whether UC patients that drink alcohol would also have increased susceptibility to infection. Our results showed mice receiving \textit{C. rodentium} following alcohol and DSS-induced colitis had decreased survival and increased weight loss, colon shortening, histopathology and clinical scores, and inflammation. Reappearance of UC symptoms during a flare stem from increases in intestinal inflammatory cytokines such as IFN-\textgamma, IL-1, IL-6, and TNF-\textalpha\textsuperscript{6-11}, which can directly lead to mucosal ulcerations, damage to the colonic epithelium, and crypt micro-abscesses\textsuperscript{193}. Coupled with the knowledge that alcohol itself induces increases in inflammation it follows that alcohol consumption could either perpetuate a current UC flare, such as we’ve provided evidence for above, and/or trigger entrance into UC flare via initiation of the inflammatory cascade, which requires further investigation. This inflammation induced damage to the intestine and thus intestinal defense mechanisms could have dramatic consequences to UC patients, especially in combination with alcohol. Recent studies found that alcohol could cause a dysbiosis of the intestinal microbiome, which, in turn, could alter the intestinal microenvironment making it more favorable to opportunistic pathogens\textsuperscript{82}. Therefore, it was imperative to understand the susceptibility to intestinal pathogens specifically after alcohol and DSS-induced colitis. With our low inoculate \textit{C. rodentium} in combination with alcohol and DSS-induced colitis, we were able to show increases in susceptibility to infection in DSS Ethanol treated mice. We recognize the burden of utilizing the mouse pathogen, \textit{C. rodentium}, which itself is used as a model of IBD, on top of our binge alcohol and DSS-induced colitis
model. Yet, our adaptation of using a much lower inoculate, $1 \times 10^5$ CFUs vs. $1 \times 10^{11}$ CFUs to induce true IBD symptoms, allowed us to shed light on the increased propensity for UC patients that drink alcohol to acquire intestinal infections. The idea that alcohol could act in such a way to worsen UC flare periods provides evidence to the clinician’s warning to UC patients to avoid drinking alcohol.

A recent mouse study showed IL-22 mediated Reg3γ synthesis prevented colonization by antibiotic-resistant *Enterococcus faecium* via Toll-like receptor (TLR)-7 stimulation on dendritic cells$^{194}$, and others have demonstrated that IL-22 mediated *Clostridium difficile* clearance from peripheral organs by upregulating systemic C3 complement activity$^{195}$. Future work in our laboratory will concentrate on combining our model of *C. rodentium* infection in UC and binge alcohol with our recombinant IL-22 or *Lactobacillus delbrueckii* treatment. This will allow us to determine whether IL-22 or probiotic treatment *Lactobacillus delbrueckii* are sufficient in restoring intestinal barrier defense against invading pathogens in the context of UC and binge alcohol as assessed by increasing intestinal epithelial cell proliferation, AMPs, and anti-inflammatory cytokines such as IL-10.

**Limitations**

We see several major limitations to our current studies. First is the inability to obtain patient tissue samples. While we would benefit greatly from studying the effects of alcohol and colitis in human intestine samples, our ability to receive proper control specimens would require physicians to take intestinal biopsies, or even perform non-invasive procedures such as colonoscopies on healthy individuals. The closest we are currently able to get to directly studying the effects of alcohol and IBD in human patient populations is through a retrospective analysis our laboratory conducted of patients admitted for IBD with a documented history of
alcohol use. Using the International Classification of Diseases, 9th Revision, Clinical Modification, (ICD-9-CM) diagnosis codes, we defined three study groups: IBD, UC, and CD. ICD-9-CM codes were also used to determine alcohol use. Within this study, we found that alcohol negatively impacts clinical outcomes of patients with IBD, specifically increased intestinal infections, antibiotic injections, abdomen CT scans, and large intestine biopsies. Examination of intestinal infection diagnoses during the admission for either the IBD, UC, or CD study groups revealed that patients who were coded for alcohol use had significantly increased intestinal infections. The IBD + alcohol patients had increased Clostridium difficile intestinal infection, poorly defined intestinal infection, and overall intestinal infections. The UC + alcohol patients had increased poorly defined intestinal infections, while the CD + alcohol patients had increased intestinal infections of all types. We would like to note that to be coded for an ICD-9 diagnosis for an alcohol related disorder, the patient must have had some history of significant alcohol intake, thus our + alcohol study group likely comprises mostly heavy drinkers. Unfortunately, the databases do not contain information regarding the amount of alcohol a patient had prior to an admission. Furthermore, the databases do not contain the exact cause of the IBD admission, thus we could not discern whether a patient was admitted for a flare or other complication of IBD. Therefore, the findings of this analysis warrant additional research of IBD patients that could provide more detailed information beyond what is available through ICD-9 codes.

In addition to the lack of patient samples, a major limitation to the progress of our understanding how the microbiome may influence the intestinal epithelial barrier and/or intestinal immune cells. The role of the microbiome, and the ability to understand the contributions of individual species of bacteria to disease has largely been feasible due to experiments carried out in germ-free facilities. Another potential experiment to study the
interaction between microbiome and gut pathophysiology is to perform fecal transplant from mice following DSS-induced colitis and binge alcohol into naïve germ free mice to see if intestinal pathology would manifest in the absence of (i.e. just due to changes in the microbiome alone). Additionally, colonizing mice with a single species of bacteria, or a labeled species of bacteria would allow us to not only tease apart the influence of specific bacterial species following acute trauma, but also monitor the translocation of microbes from the gut and how that may directly influence other organs. We hope to establish collaborations in the future that may make performing these types of experiments possible in our model.

We also would like to acknowledge that some of the experiments in this dissertation (specifically in chapter 6) have only been performed once. While we have carried out these experiments with an acceptable number of animals to run our statistical analyses, we are currently repeating these studies to verify our results before submitting these data to peer-reviewed journals.

Finally, we would like to address the use of male mice in our studies. We recognize that by only using male mice, we are missing how males and females may respond differently to colitis and binge alcohol. The largest reason for the absence of female mice from our studies is due to the need to align the estrous cycles of all the female animals involved in the study. High estrogen and progesterone levels during women’s menstrual cycles are known to increase severity of UC, and this is a mechanism that should be studied in future work. New guidelines from the National Institutes of Health requiring both males and females to be used in experimental studies will allow for our laboratory to begin work on this mechanism.
Conclusions

We have just begun to scratch the surface of studying alcohol in the context of UC. However, together, the current studies have bridged a significant gap in our current understanding of the effects of alcohol on UC pathophysiology in the intestines (Figure 28). Our work has shed light onto the physiologic, cellular, and molecular alterations to the intestinal barrier following DSS-induced colitis and alcohol. Additionally, our work has provided a potential therapeutic outlet using IL-22 or the probiotic, *Lactobacillus delbrueckii*, which showed pre-clinical efficacy in our mouse model. We have explored the role of the Jak/STAT signaling pathway using transgenic VillinCre STAT3 knockout mice to show the importance of this pathway in protection from alcohol induced exacerbated symptoms of UC. Finally, we have contributed significant advances in determining that alcohol can increase susceptibility to infection with our *C. rodentium* model. Future studies will certainly build on the foundation of this work and will further contribute to our understanding of how alcohol perpetuates UC flare.
Figure 38. Schematic of Major Findings Following Binge Alcohol and DSS-Induced Colitis With and Without rIL-22 or Lactobacillus delbrueckii Treatment. Treatment with IL-22 or Lactobacillus delbrueckii, leads to downstream activation of STAT3 (yellow), which results in intestinal barrier protection through potentially increasing AMP expression, reducing microbial dysbiosis, increasing mucus production, increasing anti-inflammatory cytokines, and promoting IEC proliferation to initiate entrance into UC remission (bottom panel).
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